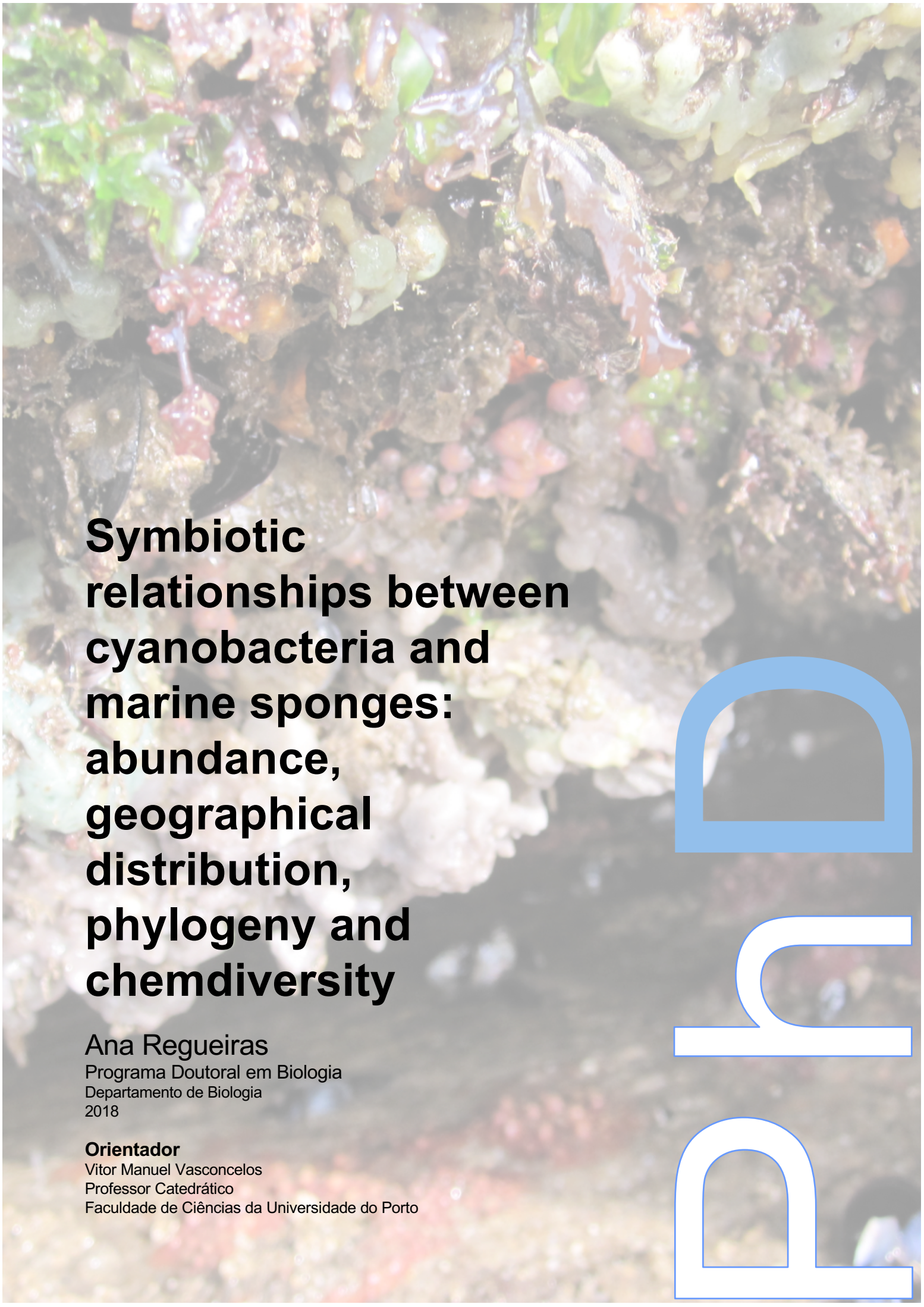

Symbiotic relationships between cyanobacteria and marine sponges: abundance, geographical distribution, phylogeny and chemdiversity

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“Eis aqui, quase cume de cabeça
De Europa toda, o Reino Lusitano,
Onde a terra se acaba e o mar começa”

Luís de Camões (Os Lusíadas)

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Abstract

Sponges are important components of marine communities, with different microorganisms being part of the sponge microbiota, with benefits both for the host and the symbionts. Due to the relation between sponges and their microbial community they must be seen as a metaorganism and studied together. Marine sponges and cyanobacteria have a long history of co-evolution with documented genome adaptations in cyanobionts. Both organisms are known to produce a wide variety of natural compounds. The coast of Portugal has some particular biogeographic circumstances, with climatic influences from the Mediterranean Sea and the Atlantic Ocean, already known to be a hotspot for marine invertebrate diversity. Also, due to eutrophication and climate change, the occurrence and diversity of marine cyanobacteria seems to be growing.

In the present thesis, when possible, it was tried to employ a multidisciplinary approach to complement each task.

Firstly (chapter 2), it was addressed the diversity of intertidal sponges from the western coast of Portugal, identifying the most common ones using an integrative approach (morphological, ecological and molecular parameters). Also, a collection of all available literature on marine sponges was made (appendix I). A comprehensive list of the intertidal species described so far are here presented, where both Calcarean and Demosponges were identified. Intertidal sponges belonging to the Class Calcarea were here identified for the first time. Demospongiae were the most common. High diversity of intertidal sponges was found, with the demosponge *Hymeniacidon perlevis* present at all sample locations. Due to its geographical distribution and abundance, *H. perlevis* was the chosen sponge for other studies here presented.

In the second study (chapter 3) the aim was to assess the cyanobacterial community associated with *H. perlevis.* As many cyanobacteria associated with sponges are known to be difficult to isolate a multidisciplinary approach was used, combining isolation and assessment through molecular methods (DGGE, cloning and sequencing). Analysis of DGGE banding pattern showed differences between sponge tissue and seawater. *Cyanobacteria* belonging to the genera *Synechococcus*, *Cyanobium*, *Synechocystis*, *Nodosilinea*, *Pseudanabaena* and *Phormidesmis* were successfully isolated, and sequencing from DGGE banding pattern revealed also *Synechococcus*, *Acaryochloris* and *Prochlorococcus*. Due to phylogenetic similarity between isolated cyanobacteria and free-living cyanobacteria, is here highlighted the importance of the use of sponges as a

source for obtaining cyanobacteria present only in small amount in seawater, as through filter-feeding they can concentrate microorganisms in their interior.

Sponges could be a good animal model for several studies but is known to be difficult to maintain *ex situ*. Through NGS analysis (chapter 4) it was assessed the diversity of bacteria associated with *H. perlevis* from natural environment and compared it through a period of 30 days under laboratory maintenance. Proteobacteria, was the major phylum present in this sponge and prevailed during the experiment. Cyanobacteria almost disappeared from sponge tissue after the 30 days, which was also confirmed through TEM analysis. We hypothesized that sponge viability was compromised by the loss of cyanobionts. This work shows the need to study the community and its balance prior to conduct more extensive studies and further investigations on how sponges are dependent on their cyanobionts must be made.

Free living cyanobacteria have been the focus of many studies aiming to address secondary metabolite production as a source of novel natural compounds. Since there are known adaptations on cyanobionts genomes, the aimed of chapter 5 was to address the toxicological potential of cyanobacterial strains isolated from marine sponges through a series of ecologically-relevant bioassays. Both the acute toxicity assay using nauplii of *Artemia salina*, and the bioassay with *Paracentrotus lividus* showed organic extracts to be more toxic than aqueous ones, especially for picocyanobacterial strains. Free-living cyanobacterial strains from other studies have shown to have the aqueous extracts with higher toxicity, showing the importance of the study of the compounds produced from the present work.

Keywords

Marine Sponges, Cyanobacteria, Diversity, Phylogeny, Portuguese coast, North-east atlantic, symbionts

Resumo

Esponjas (Porifera) são importantes membros das comunidades marinhas, que vivem em associação com diferentes microrganismos. Tanto os simbiontes como o hospedeiro são beneficiados nestas relações, e devido ao grau de associação, ambos devem ser assumidos como um metaorganismo. Esponjas e cianobactérias têm uma longa história de coevolução, tendo já sido documentadas adaptações genómicas nas cianobactérias simbióticas. Ambos são produtores de inúmeros compostos naturais.

A costa de Portugal possui circunstâncias geográficas muito particulares, com influências tanto do Mediterrâneo como do Atlântico. Estas particularidades fazem desta zona um "hotspot" de diversidade de invertebrados marinhos. Eutrofização e alterações climáticas têm aumentado tanto a ocorrência, como a diversidade de cianobactérias marinhas.

No presente trabalho, sempre que possível, foram utilizados métodos multidisciplinares. No capítulo 2 o objetivo foi identificar as esponjas intertidais mais comuns presentes na costa oeste de Portugal usando parâmetros ecológicos, morfológicos e uma análise molecular. Foi também feita uma extensa análise bibliográfica das espécies descritas em Portugal (apêndice I). As espécies identificadas pertencem às Classes Calcarea e Demospongiae. Membros intertidais da Classe Calcarea foram descritos aqui pela primeira vez. O presente estudo mostrou a existência de uma extensa variedade de esponjas, sendo que a espécie *Hymeniacidon perlevis* foi a mais comum. Devido à sua distribuição geográfica e abundância, *H. perlevis* for a espécie selecionada para vários ensaios seguintes.

No capítulo 3 a comunidade de cianobactérias associadas à esponja H. perlevis foi investigada usando tanto métodos convencionais de isolamento e cultura, como metodologias moleculares (DGGE, clonagem e sequenciação). A análise dos padrões das bandas mostrou a comunidade de cianobactérias associadas às esponjas por diferir da presente na amostra de água. Cianobactérias dos géneros *Synechococcus*, *Cyanobium*, *Synechocystis*, *Nodosilinea*, *Pseudanabaena* e *Phormidesmis* foram isoladas e a diversidade foi complementada com a informação proveniente da análise molecular, onde foram detectados os géneros *Synechococcus*, *Acaryochloris* e *Prochlorococcus.* As estirpes isoladas mostraram ser muito semelhantes a estirpes anteriormente isoladas e de vida livre, mostrando que as esponjas poderão ser uma boa fonte para obtenção de cianobactérias, devido à sua capacidade de filtração e acumulação dos microrganismos no seu interior.

Devido à sua posição filogenética, as esponjas poderão tornar-se em bons modelos animais para diversos estudos. Para tal, manutenção *ex situ* é imperativa. Usando uma análise por sequenciação de nova geração (capítulo 4) estudou-se a diversidade de bactérias associadas à esponja *H. perlevis*, a qual foi comparada com a comunidade de bactérias após manutenção em laboratório (30 dias). O Filo Proteobacteria foi o principal, mantendo-se em todas as amostras de esponjas. As cianobactérias quase desapareceram da esponja após manutenção em laboratório e pouco depois a esponja perdeu viabilidade, morrendo. Por análise TEM foram identificadas cianobactérias em vacúolos especializados tanto para o tecido da esponja in situ, como após 15 dias *ex situ*. Ao fim dos 30 dias não foram identificadas cianobactérias. Possivelmente, a perda de cianobiontes interferiu com a viabilidade da esponja. Este trabalho mostrou como o balanço na comunidade bacteriana pode afetar a viabilidade da esponja, mostrando a necessidade de um estudo mais aprofundado para determinar o verdadeiro papel dos cianobiontes nesta esponja.

Cianobactérias marinhas de vida livre têm sido o alvo de inúmeros estudos para detectar novos compostos secundários bioativos. Uma vez que os cianobiontes podem possuir adaptações genômicas, o seu potencial como produtor de novos compostos está ainda por explorar. No capítulo 5 o potencial toxicológico de estirpes isoladas de esponjas marinhas foi estudado. Os estratos orgânicos destas estirpes, especialmente das estirpes picoplanctónicas mostraram ser os mais tóxicos tanto no bioensaio agudo de *Artémia salina*, como no do equinoderme *Paracentrotus lividus*. Estudos realizados com estirpes de vida livre têm demonstrado os estratos aquosos como mais tóxicos, quando comparados com os orgânicos, contrastando com os resultados aqui apresentados, e demonstrando o potencial, e a necessidade de explorar estes novos compostos.

Palavras-chave

Esponjas marinhas, cianobactérias, diversidade, filogenia, costa portuguesa, atlântico nordeste, simbiontes

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List of Abbreviations

%	percentage
‰	per mille
<	less than
Abs	absorbance
AICc	Akaike's information criterion with correction
ANOVA	Analysis of variance
Ara-A	9-β-D-arabinofuranosyladenine
Ara-C	cytosine arabinoside
AZT	azidothymidine
BI	Bayesian inference
BLAST	Basic Local Alignment Search Tool
BLASTn	nucleotide BLAST
bp	base pairs
CaCl2	Calcium Chloride
CH2Cl2:MeOH	dichloromethane and methanol mixture
chl *a*	chlorophyl a
chl *d*	chlorophyl d
CIIMAR	Centre of Environmental and Marine Research
cm^3	cubic centimetre
CO1	Mitochondrial cytochrome oxidase subunit 1
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
e.g.	for exemple
$E/m^{-2}s^{-1}$	Einstein per metre sqare per second
EDTA	Ethylenediaminetetraacetic acid
ERDF	European Regional Development Fund
FCT	Foundation for Science and Technology
FDA	U.S. Food and Drug Administration
FISH	Fluorescence *in situ* hybridization
g	grams
g	gram
g	g force
GC	Guanine cytosine
gDNA	genomic DNA
GTR+G+I	general time-reversible plus gamma distributed plus invariant sites
h	hour
HEMS	Histology and Electron Microscopy Service
HIV/AIDS	Human immunodeficiency virus infection and acquired immune deficiency syndrome
HKY+I+G	Hasegawa-Kishino-Yano plus invariant plus gamma distributed
HMA	High microbial abundance
IBMC	Institute for Molecular and Cell Biology
INNOVMAR	Innovation and Sustainability in the Management and Exploitation of Marne Resources
kg	kilogram
km	kilometre
kV	kilovolt
L	litre
LEGE	Laboratory of Ecotoxicology, Genomics and Evolution
LEGE CC	LEGE culture colection

LMA	Low microbial abundance
m	meters
M	molar
mg	miligram
MgCl2	magnesium chloride
min	minute
mL	milliliter
ML	Maximum Likelihood
mM	milimole
mm	milimitre
mo	month
Myr	Million years
N	North
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NE	Northeast
ng	nanogram
NGS	Next-generation sequencing
nm	nanometer
NNI	nearest-neighbor-interchange
ºC	Celsius degrees
OTU	Operational taxonomic units
PBS	Phosphate-buffered saline
PCoA	Principal Coordinate Analysis
PCR	Polymerase chain reaction
QIIME	Quantitative Insights Into Microbial Ecology
rDNA	ribossomal DNA
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	Ribosomal RNA
s	seconds
S.chao1	Expected richness with Chao1 estimator
S.obs	Observed species richness
SD	standard deviation
TAE	Tris-acetate-EDTA
TEM	Transmission electron microscopy
TM	Trade mark
TrN+I+G	Tamura-Nei plus invariant plus gamma distributed
U	units
U.V.	ultraviolet
UniFrac	Unique Fraction method
V	volts
v/v	volume per volume
W	West
w.w.	Wet weight
w/v	weight per volume
yr	year
µg	microgram
µL	microlitre
µm	micrometre
µmol	micromol

Chapter 1. Introduction

Porifera

Sponges are ancient animals (with fossil records dating back to around 580 million years (Myr))(Hentschel et al., 2006), belonging to the Phylum Porifera, and constitute the bottom (less evolved) of the Metazoan branch. Love et al. (2009) also found chemical fossil records from marine demosponges from around 635 Myr ago. With a simple body plan, highly totipotent cells, a characteristic aquiferous system and different reproduction strategies, Porifera lifestyle has proven to be very successful. Among the 28 aquatic phyla, sponges are the ones with greater diversity in terms of number of species and morphological characters (Hooper & van Soest, 2002). They contributed to the construction of the reefs and to the increase of ocean diversity and are one of the most abundant groups of animals (Hooper & van Soest, 2002). Recent studies also point to their role in the increase of oxygen on the oceans, a requisite for the explosion of more complex life forms on Earth (Lenton et al., 2014). Sponges are important organisms playing in marine environments crucial steps of the cycle of dissolved nutrients and organic matter (Maldonado & Riesgo, 2008), and are a vast source of compounds with biotechnological applications (Leal et al., 2012). These and other roles were already subjected to reviews as the one made by Bell (2008).

Sponges are sessile, exclusively aquatic organisms, presented in marine and freshwater environments, from tropical to temperate and polar areas, occurring at all depths (Sarà & Vacelet, 1973, Bergquist, 1978, Van Soest et al., 2012), with an enormous variety of shapes and colour. In benthic environments they can occupy as much as 80% of substrate (Webster & Thomas, 2016). Sponges can have sexual or asexual reproduction. Asexual reproduction occurs through fragmentation, budding, or gemmule production. Without true tissues or organs, sponges are constituted by cells that maintain their totipotency, and that are more or less specialized to maintain vital functions (Hooper & van Soest, 2002). They survive by filtering water to obtain food particles and oxygen. As represented in Figure 1-1, water enters through the ostia, which is capable of opening and close, as well as to regulate the diameter of the pore, then goes through an internal system of canals and chambers surrounded by specialized flagellated cells, the choanocytes, that are responsible for the generation of a water current. Finally, water is expelled through the osculum. Between the canals and chambers there is a collagenous matrix, called mesohyl, responsible for harbouring different cells, like the archeocytes (amoeboid totipotent cells capable of moving freely, involved in digestion, transport of products through the sponge body, and excretory activities) and to support fibbers and structures from the skeleton (Van Soest et al., 2012).

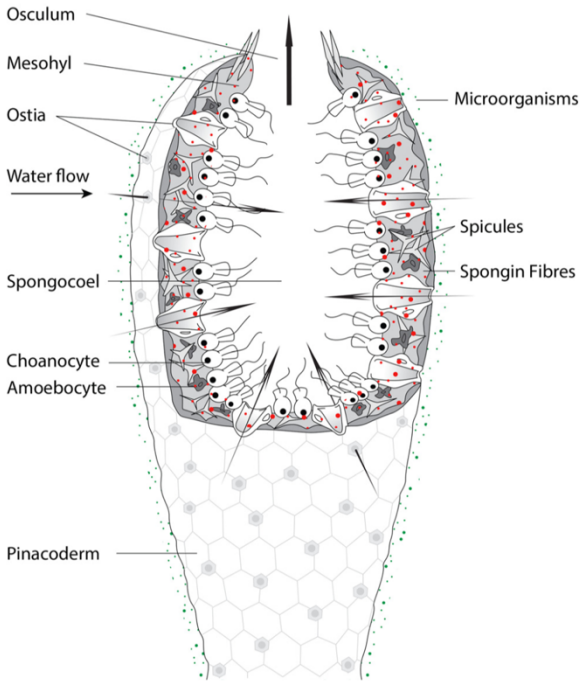

Figure 1-1. Schematic representation of a marine sponge (asconoid sponge). Environmental microorganisms are represented in green and symbiotic microorganisms are in the mesohyl, represented in red. Extracted from Webster and Thomas (2016).

As filter feeders, they are capable of filtering thousands of litters of water per day (Hentschel et al., 2006), using microorganisms as their main source of food (Hardoim et al., 2009) and during this process, some microorganisms survive in the mesophyll tissue and can be established as part of the sponge-specific microbiota (Kennedy et al., 2007) (Figure 1-1). These microorganisms may comprise as much as 40% of the total sponge volume (Vacelet, 1975, Vacelet & Donadey, 1977, Webster & Taylor, 2012). Within the symbiotic microorganisms are bacteria, fungi, unicellular algae and cyanobacteria (Webb & Maas, 2002, Taylor et al., 2007a). The variety of microorganisms in sponges, as well as the compounds produced by these associations made sponges the centre of various studies.

The body is supported by collagenous fibbers, spongin fibbers and/or an inorganic skeleton made of silica or calcium carbonate (spicules) that can be absent (Hooper & van Soest, 2002, Van Soest et al., 2012). There are more than 8500 species (according to World Porifera Database (Van Soest et al., 2017)) of Porifera accepted and around 2300-3000 specimens already collected but undescribed (Appeltans et al., 2012) and is divided into 4 Classes: Homoscleromorpha, Calcarea, Hexactinellida and Demospongiae. Calcarea comprehends exclusively marine species with a mineral skeleton entirely of calcium carbonate and with around 800 described species. Hexactinellida are called glass sponges and have a siliceous skeleton with 6 rayed

spicules. Normally occur in deep waters and there are around 600 different species described. Demospongiae have siliceous spicules and/or spongin fibbers. Spicules can be absent. They comprise about 83% of all living sponges (Van Soest et al., 2012, Morrow & Cárdenas, 2015) and are mainly marine but also occur in freshwaters.

Sponge classification rely greatly in spicules morphology and arrangement in sponge tissue (Morrow et al., 2013). As sessile animals, with only a small part of life with mobility (larvae stage) and the occasional asexual reproduction most species are specific to a regional location, with several endemisms (Van Soest et al., 2012). Sponge morphology has a high degree of plasticity, with variations not only between different species, but also within the same species, because of environment factors, such as sedimentation, hydrodynamics, light, turbidity, substratum type and angle and flow regime (Bell & Barnes, 2000, Van Soest et al., 2012). Also, many of these morphological characters can be non-homologous (Boury-Esnault, 2006) resulting in unresolved and ambiguous classification. Identification problems resulted in disregarding sponges in large-scale surveys. To overcome this problem, many studies have been using an integrative approach, combining morphological and molecular characters to identify sponges. Phylogenetic studies have shown that the four porifera classes are monophyletic, but many major clades of sponges appear to be paraphyletic, leading to a revision of traditional sponge classification (Cárdenas et al., 2012, Hill et al., 2013, Thacker et al., 2013).

There are two main commercial interests on sponges. Their use as bath sponges and as a source of bioactive compounds with pharmaceutical and/or toxicological interest. These compounds are produced by sponges and/or their associated microorganisms and constitute a major contributor to sponges success. In an ecological perspective, sponges can also be used as bioindicators of water quality or, due to their simple body plan and early-branching position in the metazoan tree of life, as an animal model for scientific studies, being used for animal phylogenetic, neuronal and morphological evolution.

The coast of Portugal has some particular biogeographic circumstances, receiving climatic influences from the Mediterranean Sea and the Atlantic Ocean. As a result, biodiversity is a mixture of the one present in the North-eastern Atlantic coasts and the Mediterranean (Boaventura et al., 2002). Though sponges can be dominant members of some communities and play important roles in a variety of ecosystem functions (Rützler, 2012, Wulff, 2012), our knowledge of the intertidal and subtidal marine sponges in Portugal derives from the works of Carter (1876), Hanitsch (1895), Lévi and Vacelet (1958), Pérès (1959), Saldanha (1974), Lopes and Boury-Esnault (1981), Monteiro

Marques et al. (1982), Monteiro Marques (1987), Lopes (1989), Araújo et al. (1999), Naveiro (2002), Pereira (2007), Pires (2007), Costa (2012). Analysing the bibliography previously described (see comprehensive tables in appendix I), it is possible to see that the majority of sponge species identified in Portugal are subtidal (Figure 1-2a). Most of them belong to the class Demospongiae (Figure 1-2b) and within it to the Subclass Heteroscleromorpha (Figure 1-2c). The coast of Portugal has an enormous diversity of sponges, with more than 200 different species described (this number also includes the ones described for the first time in this work). Most studies focus only on diversity of subtidal sponges, lacking information on intertidal diversity.

In recent years, due to difficulties in sponge identification, most diversity studies neglected phylum Porifera and, improving our understanding of their biodiversity can be essential for habitats protection. For example, Peterson et al. (2006) showed that the increase of water phytoplankton blooms can be linked to a decrease of sponge populations, and not directly linked with increased nutrient intake of the ecosystem.

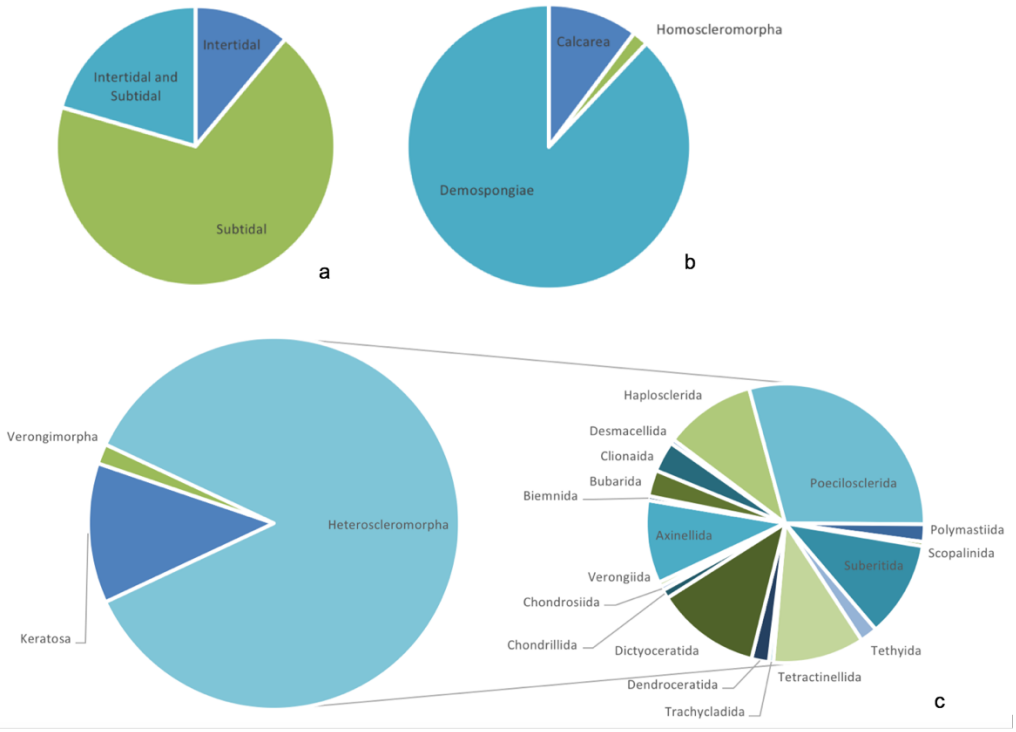

Figure 1-2. Porifera distribution in Portugal (continental) according to the literature. (a) Distribution by sampling depth; (b) distribution by Porifera Class; (c) distribution by Subclass of the Class Demospongiae and by Orders of the Subclass Heteroscleromorpha.

Cyanobacteria

Cyanobacteria are prokaryotic photosynthetic organisms, with a high morphological, physiological and metabolic diversity. Fossil record point to the existence of cyanobacteria dating back 3.5 billion years ago, with very little morphological changes until today (Adams & Duggan, 1999). According to Codd et al. (2016), cyanobacteria are responsible for the creation of the Earth's aerobic atmosphere, continuing to be crucial elements in biological cycling of carbon, nitrogen, and minerals. Cyanobacteria are primary colonisers, being present in almost all ecosystems, including fresh, brackish, and marine waters, and also rocks and soils, as well as extreme environments (Codd et al., 2016).

Cyanobacteria are prokaryotes with their nomenclature ruled both by the International Code of Nomenclature for Algae, Fungi and Plants and the International Code of Nomenclature of Prokaryotes, emerging different types of systematics. This issue has been addressed in more depth by Ramos et al. (2017). In recent years, cyanobacterial taxonomy was under revision, with a new proposal made by Komárek et al. (2014).

Cyanobacteria form symbiotic relationships with numerous eukaryotic organisms such as plants, fungi and animals (Adams, 2000). In the marine environment occur with sponges, ascidians, echuroid worms, diatoms, dinoflagelates and protozoans (Carpenter & Foster, 2002). In sponges, cyanobacteria are important photosynthetic symbionts. Host sponges with cyanobionts can comprise up to 30-50% of the sponges on tropical reefs (Rützler, 1990, Burja & Hill, 2001, Erwin & Thacker, 2007) and 45-60% in temperate waters (Lemloh et al., 2009). As photoautotrophic and sometimes heterotrophic, capable of fixing nitrogen, they can provide the host both nitrogen and dissolved organic carbon (Adams, 2000, Carpenter & Foster, 2002), and some hosts are even unable to survive without these symbionts (Thacker, 2005).

Their secondary metabolism is very active, known for being one of the most rich and diverse sources of compounds not only toxic, but also with pharmacological (e.g. anticancer, antibiotic and anti-inflammatory properties) and industrial interests (e.g. biofertilizers and anti-fouling properties). In marine environments, cyanobacteria are a recognized source for novel metabolites, with hundreds of different compounds discovered, mainly from filamentous and tropical cyanobacteria.

The first interest in cyanobacteria secondary metabolites came from their ability to produce toxins. These toxic compounds are chemically very diverse (Codd et al., 2016) and can cause a variety of symptoms, acting as hepatotoxins, neurotoxins, cytotoxins, dermototoxins and irritant toxins (Wiegand & Pflugmacher, 2005).

Marine cyanobacteria diversity on the Portuguese coast have already been the focus of various studies (e.g. Brito et al. (2012), Leão et al. (2013)), with *Cyanobium*, *Leptolyngbya* and *Pseudanabaena* as the most abundant genera among isolates (Brito et al., 2012). A huge collection of isolated strains from the coast of Portugal are deposited in LEGE culture collection (LEGE CC) (Ramos et al., 2018). These isolated strains were found to be a source of bioactive compounds (Leão et al., 2013, Costa et al., 2014, Brito et al., 2015, Costa et al., 2015, Afonso et al., 2016) namely strains from the genera *Cyanobium* (Costa et al., 2015), *Leptolyngbya*, *Synechocystis*, *Nodosilinea* and *Pseudanabaena* (Costa et al., 2014, Afonso et al., 2016).

Sponges and their microbial community

In the present work symbiosis will be used according to the definition from de Bary (1879): two organisms of different species that live together in association (mutualistic or commensal, but not parasitic), over a long period of time.

Marine sponges are known for harbouring diverse symbiotic microorganisms, with mutual benefits both for the host and the partner. These associations evolved millions of years ago and played an important role in sponge survival and evolution (Taylor et al., 2007b).

The majority of microorganisms inhabit the mesohyl matrix (Vacelet & Donadey, 1977), namely heterotrophic and autotrophic bacteria (Hentschel et al., 2003). In the mesohyl bacteria can also appear inside bacteriocytes (Vacelet & Donadey, 1977) or in vacuoles. Vacelet (1970) have also found some bacteria in the nuclei of certain sponge cells, appearing to be correlated with a pathogenic association. Photosynthetic bacteria (cyanobacteria and eukaryotic algae) are often located in light-exposed tissue layers, as the outer layer (Rützler, 1985, Wilkinson, 1992).

The first studies in this area date back from the 70's. Reiswig (1971) was the first to address the existence of microorganisms within sponge tissue, pointing to bacterial cells being consumed by sponges. The first works addressing the associations between sponges and microorganisms were from Vacelet and Donadey (Vacelet, 1970, Vacelet, 1971, Vacelet, 1975, Vacelet & Donadey, 1977) and from Wilkinson (Wilkinson, 1978a, Wilkinson, 1978b, Wilkinson, 1978c). Vacelet and Donadey (1977) using electron microscopy showed the existence of intact bacterial cells in the mesohyl and were also able to identify different sponges morphotypes harboured different amounts of bacteria, where massive sponges with a dense mesohyl had many bacteria, and sponges with a smaller mesohyl and well-irrigated had almost none bacteria. According to Vacelet and Donadey (1977), bacteria could account up to 38% of sponge wet weight (w.w.). The same bacterial morphotypes were later also identified in the works of Wilkinson (Wilkinson, 1978a, Wilkinson, 1978c). Wilkinson (1978b) was able to divide sponges into 6 different clusters, according to bacterial diversity similarity between sponge tissue and the surrounding water. Wilkinson (1978b) showed that some sponges had bacterial communities completely different from the ones present in the surrounding water and characterized them as strictly symbionts.

The first studies of the microbial community assessment in sponges used culture dependent techniques, being able to recover up to 11% of total bacterial within sponge tissue (Santavy et al., 1990, Friedrich et al., 2001, Hentschel et al., 2006, Sipkema et al.,

2011). According to Hentschel et al. (2006) Proteobacteria, especially alpha- and gamma- are the majority of cultivated bacteria.

Later, the use of molecular based techniques uncovered the existence of many more phyla, allowing to overcome issues related to culture dependent techniques. The use of fluorescence *in situ* hybridization (FISH) allowed the detection of single cells, and to identify their phylogeny, location and morphology (Hentschel et al., 2003). Denaturing gradient gel electrophoresis (DGGE) allowed fingerprinting of bacterial communities. This technique gives insights in microbial diversity and provides the ability to track changes in the community over time or space (Hentschel et al., 2003). 16S rDNA library construction was the most informative technique of this three, phylogenetically speaking (Hentschel et al., 2003). One of the first studies of this molecular era was performed by Hentschel et al. (2002) comparing the microbial community between sponges, surrounding water and sediment. This study showed for the first time the evidence of a monophyletic, sponge specific clusters and a uniform bacterial community in marine sponges on a global scale. This "specific clusters" were explained through vertical transmission, where bacterial cells are pass from sponge to offspring through reproductive cells (Hentschel et al., 2002). Evidences of vertical transmission were found by Sharp et al. (2007), Schmitt et al. (2007), Usher et al. (2001), Sipkema et al. (2015), who retrieved different bacterial phyla from both adult sponges and offspring.

The construction of 16S rRNA gene libraries by PCR or DGGE allowed the identification of the following phyla: Acidobacteria, Bacteroidetes, Chlamydae, Chloroflexi, Cyanobacteria, Deferribacteres, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadites, Lentisphaerae, Nitrospira, OP10, OP11, Planctomyces, Proteobacteria (alpha, beta, delta, epsilon and gamma), Spirochaetes, Tenericutes, TM6, TM7, Verrucomicrobia and WS3 (Taylor et al., 2007a, Webster & Taylor, 2012). A candidate phyla, "Poribacteria" was discovered by Fieseler et al. (2004), being almost exclusively associated with sponges and only scarcely found in seawater (Taylor et al., 2013). The use of molecular techniques showed that bacterial communities present in marine sponges were very different from the ones present in the surrounding water as pointed by Wilkinson (1978b) (Hentschel et al., 2006, Taylor et al., 2007a, Hardoim et al., 2009, Hentschel et al., 2012, Webster & Taylor, 2012).

The use of high-throughput sequencing techniques such as next generation sequencing (NGS) 454-pyrosequencing provided new insights in sponge microbiology. Lee et al. (2011) concluded that bacterial communities in sponges were species specific and Schmitt et al. (2012) in a study using 32 marine sponges collected worldwide found the existence of 16 different bacterial phyla and a low core community (<1%). Both works

highlighted the idea of a species-specific microbial community, going against the idea of a worldwide sponge specific community across different species. NGS studies unveiled many different phyla (Cárdenas et al., 2014, Hardoim et al., 2014, Kennedy et al., 2014, Naim et al., 2014). Thomas et al. (2016), as part of the global sponge microbiome project, studied 81 different sponge species worldwide collected, founding the existence of 41 microbial phyla and candidate phyla. The overall patterns of microbial diversity were also found in the work of Moitinho-Silva et al. (2017) in a study comprising 268 sponge species. Just like the previous works, most OUT's (operational taxonomic units) were present in a small fraction of the sponges, and only a few were found in most sponge species (Moitinho-Silva et al., 2017). Although recent 454 pyrosequencing studies revealed many new microbial phyla in sponges, it also showed that the dominant bacterial taxa were the same as the ones described in previous studies using 16S rRNA gene libraries. As described by Pita et al. (2018) and represented in Figure 1-3, the most dominant bacterial phyla are: Proteobacteria (Gamma- and Alpha-), Actinobacteria, Chloroflexi, Nitrospirae, Cyanobacteria and Candidatus Phylum Poribacteria.

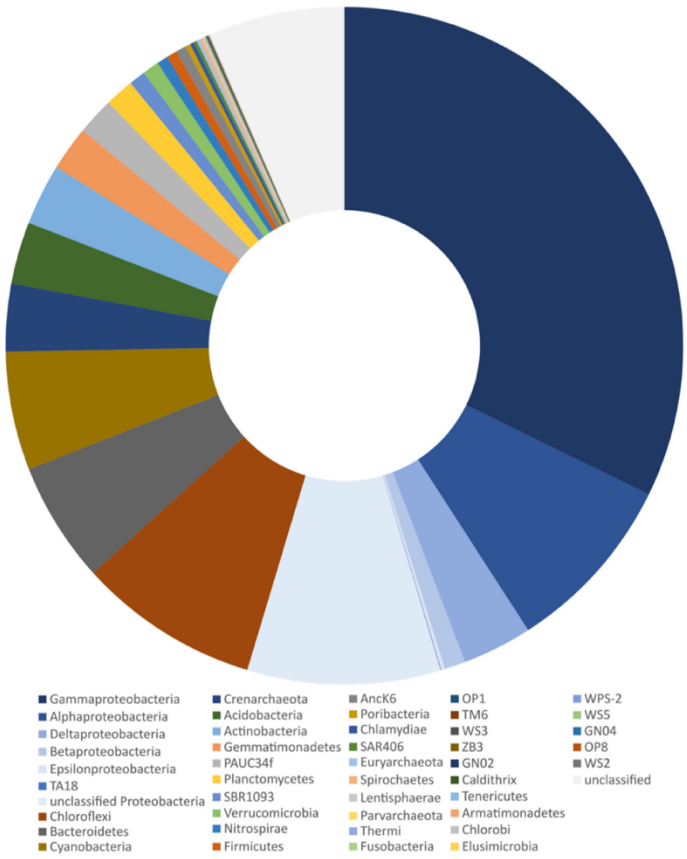

Figure 1-3. Scheme from the work of Pita et al. (2018): "Microbial OUT richness in sponge-associated microbial communities at phylum level. The Greengenes annotation of the representative sequences for sponge-associated OTUs detected by the Global Sponge Microbiome (Thomas et al., 2016) was used to create this chart. A diversity of 43,034 OTUs from 39 classified microbial phyla (Bacteria and Archaea) was detected in the microbiomes of the 81 species in this project (Thomas et al., 2016)".

According to the bacterial community abundance, sponges were classified into two categories: high microbial abundance (HMA) and low microbial abundance (LMA) sponges (Hentschel et al., 2006). HMA sponges have 10^8-10^{10} bacteria per gram of sponge (w.w.) (Friedrich et al., 2001, Hentschel et al., 2006, Weisz et al., 2007), corresponding to 2-4x more bacteria than seawater. In this sponges, also known as "bacteriosponges", microorganisms can account for as much as 40-60% of sponge biomass (Grozdanov & Hentschel, 2007). LMA sponges have the same amount of bacteria than seawater (10^5-10^6 bacteria per gram of sponge (w.w.)) (Hentschel et al., 2006). Different studies have also point to a microbial diversity at phylum-level in between HMA and LMA sponges (Weisz et al., 2007, Erwin et al., 2011, Schmitt et al., 2012, Giles et al., 2013, Moitinho-Silva et al., 2014). LMA sponges are often dominated by Proteobacteria (alpha-, beta- and gamma-) or Cyanobacteria (genus Synechococcus) and lack the candidate phylum Poribacteria. HMA sponge have higher phyla as dominant, such as Proteobacteria, Chloroflexi, Acidobacteria, Actinobacteria, candidate phyla Poribacteria and other.

Sponge bacteria associations provide many benefits for the host, such as in nutritional processes through translocation of metabolites in the form of glycerol (Wilkinson & Fay, 1979), organic phosphate and nitrogen (Wilkinson & Fay, 1979) or glucose (Wilkinson, 1980), enhancing its growth rate and competitiveness with other benthic communities (Wilkinson, 1980, Arillo et al., 1993) and sponge skeleton stabilization (Wilkinson et al., 1981). Bacteria can also participate in chemical defence of the host against both predators and biofouling (Unson et al., 1994, Schmidt et al., 2000). It has also been proven that sponge survival, in many cases, can be directly linked to the stability of certain symbionts. For example, Thacker (2005), observed that a decline on the cyanobacterial community of the sponge was related with a decrease of sponge health. On the other hand, microorganisms can also benefit from these associations. The sponge provides a steady nutrient supply through filter-feeding activity. Ammonia, a metabolic end product from sponges, also provide nitrogen for the microorganisms (Hentschel et al., 2012).

Cyanobacteria and Sponges associations

Porifera and Cnidarians are the most common marine animals capable of establishing symbiotic relationships with photosynthetic microorganisms. That is due to their simple morphology and high surface/volume area, allowing photobionts to capture light (Venn et al., 2008).

Symbiotic associations of marine sponges and cyanobacteria are common worldwide, from tropical, temperate and polar ecosystems. In tropical areas, cyanosponges can comprise 30-50% of the sponge community (Rützler, 1990, Burja & Hill, 2001, Erwin & Thacker, 2007), and in temperate regions up to 64% (Lemloh et al., 2009). According to Steindler et al. (2002) cyanosponges can achieve up to 85% of all intertidal sponge communities in tropical reefs. Sponge cyanobacteria associations has been characterized as mutualistic (Konstantinou et al., 2018).

All Classes of Porifera have already been reported as having cyanobacteria symbionts, especially from the classes Demospongiae and Calcarea. Diaz et al. (2007) reported, approximately 10 years ago, the existence of more than 100 cyanosponge species and a recent new study elevates this number to more than 320 species (Konstantinou et al., 2018). This increase is probably related to the use of molecular (sequencing, DGGE) and metagenomic techniques (454-pyrosequencing and Illumina), contrasting to previous techniques used, such as chlorophyll *a* measurements, microscopy techniques (light microscopy, TEM) and isolation, which retrieved much smaller amounts of cyanobacteria diversity.

Both coccoid and filamentous cyanobacteria have been described in sponges. The majority of the studies reporting the existence of cyanobacteria haven't done a taxonomical identification beyond phylum level (Konstantinou et al., 2018). Among cyanobacteria, different species from the genera *Aphanocapsa*, *Synechocystis*, *Synechococcus*, *Prochloron* and *Oscillatoria* have been reported, but a number of unnamed cyanobacteria have been also found (Carpenter & Foster, 2002, Usher, 2008). According to Konstantinou et al. (2018), the genus *Synechococcus* is the most widely reported and studied. Isaacs et al. (2009) also found *Pseudanabaena* and *Phormidium* but weren't able to cultivate it. In Portugal, *Xenococcus*-like and *Acaryochloris* sp. were reported from the intertidal marine sponge *Hymeniacidon perlevis* (Alex et al., 2012, Alex & Antunes, 2015). Other cyanobacterial genera already identified in sponge species are *Leptolyngbya*, *Plectonema*, *Myxosarcina*, *Limnothrix* (Angermeier et al., 2011), *Lyngbya*, *Cyanothece*, *Mastigocladus*, *Anabaena*, *Calothrix*, *Microcoleus*, *Hydrocoleum* (Zhang et al., 2014), *Prochlorococcus*, *Pleurocapsa*, *Chroococcidiopsis*, *Crocosphaera*, and

Desmonostoc (Fromont et al., 2016). Usher et al. (2006) showed that geographical distinct areas and different sponges can have the same symbiont and each sponge can harbour more than one cyanobacteria species.

Molecular techniques demonstrated that cyanobionts in sponges differ from those of the seawater communities (Usher et al., 2004, Steindler et al., 2005, Lemloh et al., 2009). These techniques have been able to assess the cyanobacterial diversity among the sponge hosts (Taylor et al., 2007a). Molecular metagenomic sequencing technology have presented in the last few years with new insights in terms of cyanobacteria diversity (Wang et al., 2009, Gao et al., 2014, Burgsdorf et al., 2015, Konstantinou et al., 2018).

Among all cyanobacteria, it seems that "Candidatus *Synechococcus spongiarum*", belonging to a sponge specific lineage is the most prevalent symbiotic group (Usher et al., 2004, Steindler et al., 2005, Erwin & Thacker, 2007, Erwin & Thacker, 2008, Lemloh et al., 2009) with little genetically difference between different hosts and geographical areas (Erwin & Thacker, 2008). Molecular analysis of another common cyanobacteria, *Oscillatoria spongeliae*, showed that this is a specialist symbiont with genetically different populations according to the host sponge (Thacker & Starnes, 2003).

Unicellular and filamentous cyanobacteria can cover up to 50% of a sponge's cellular volume (Rützler, 1990). Most cyanobacteria are present intercellularly, free-living in the mesohyl (Wilkinson, 1978c), but *Aphanocapsa feldmannii*, can occur intracellularly, in specialized arqueocytes vacuoles (Rützler, 1990) named cyanocytes. Some cyanobacteria have also been found to occur in digestive vacuoles (Wilkinson, 1978c).

Cyanobacteria are photoautotrophic and, in some cases, facultative heterotrophic, providing many benefits for the host. As photosynthetically active in sponges, they transfer glycerol (Wilkinson, 1980) and organic phosphate to the host (Wilkinson & Fay, 1979), which can comprise to more than 50% of the metabolic needs of the sponge (Carpenter & Foster, 2002), enhancing sponge growth (Vacelet, 1971, Wilkinson, 1980, Rützler, 1990, Arillo et al., 1993). As cyanobacteria are capable of doing photosynthesis in low light environments, this symbiosis can occur at different depths (Usher, 2008), and due to their active secondary metabolites, cyanobacteria help in sponge defence (Carpenter & Foster, 2002), in protection from U.V light (Adams, 2000) and in substrate competition (Usher et al., 2004, Taylor et al., 2007a). They are also capable of fixing nitrogen (Adams, 2000) and help in ammonia conversion (Usher, 2008). Some sponges are uncapable of surviving without their cyanobionts (Thacker, 2005).

Cyanobacteria can also benefit from these associations. Sponges work as shelters (Erwin & Thacker, 2007), protecting cyanobacteria from extreme environmental conditions and from predation (Adams, 2000, Usher, 2008). Sponges have also better

levels of phosphorous and ammonia than sea water (Usher, 2008). Due to primary productivity and nutrient cycling enhanced by these associations, marine ecosystems can also benefit (Diaz & Rützler, 2001)

Vertical transmission seems to be the main form for cyanobacteria acquisition (Usher et al., 2001, Oren et al., 2005, Usher et al., 2005, Schmitt et al., 2007, Sharp et al., 2007). Offspring are unable to feed and the presence of cyanobacteria provides them with photosynthetic energy (Lemloh et al., 2009), enhancing its competitive fitness (Oren et al., 2005). Maldonado (2007) observed that in some sponges, symbionts were always obtained from the environment (horizontal transmission) and never present in gametes or embryos. In some cases, both transmission routes can be present (Thacker & Freeman, 2012).

Sponges and cyanobacteria as a source for novel compounds

Traditionally, plants from terrestrial environments, were the main source of natural product-derived drugs. In the early 50's researchers started looking at marine environments as a natural drug source. Since the late 80's there was a "boom" of articles reporting marine natural products as reviewed in the marine natural products reviews (Faulkner, 1986, 1987, 1988, 1990, 1991, 1992, 1993, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, Blunt et al., 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018). Sponges, among marine invertebrates, are the most prolific source of bioactive compounds (Blunt et al., 2010), as shown in Figure 1-4, comprising 48.8% of all marine natural products discovered since 1990 (Leal et al., 2012), with a wide range of natural products activities, such as antibacterial, antifungal, antitumor, antiviral, antioxidant, antifouling, among other and chemical classes (e.g. terpenoids, alkaloids, peptides and polyketides) (Blunt et al., 2005). Although these compounds have been isolated from sponges, it is now widely accepted that symbiotic microorganisms are the main producers (Hentschel et al., 2006). Actinobacteria, Cyanobacteria, Firmicutes and Proteobacteria (alpha and gamma classes) are the main phyla producing secondary metabolites in sponges (Thomas et al., 2010b).

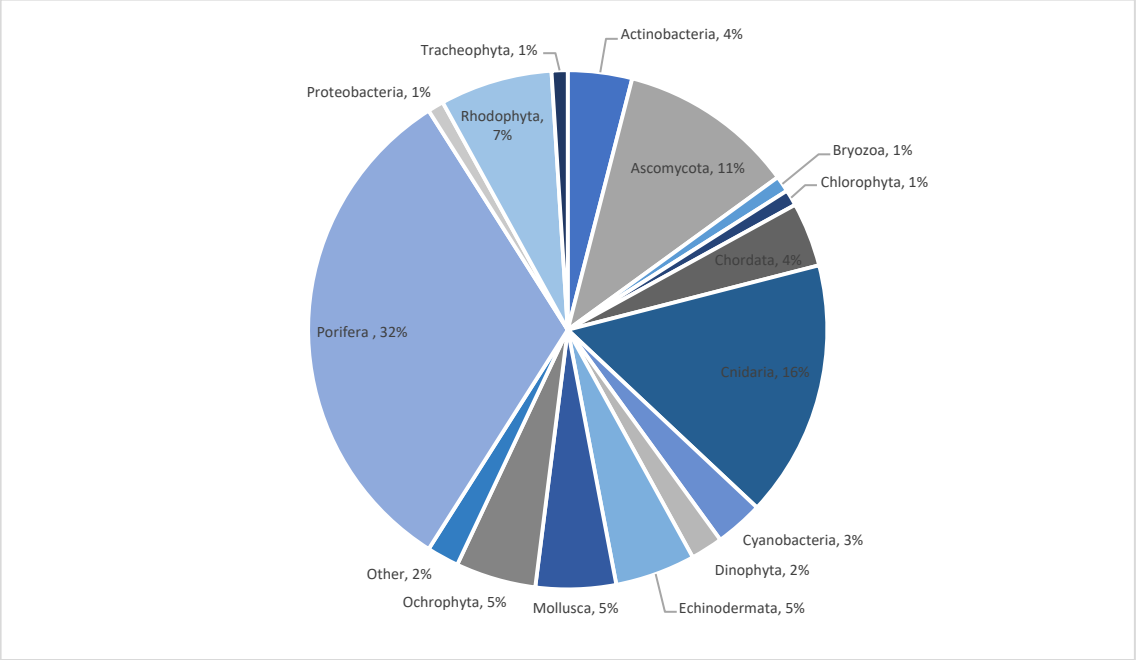

Figure 1-4. Collection effort from 1971-2015. Adapted from the work of Blunt et al. (2017)

The potential of marine environments as a source of novel compounds started in the 50's with the isolation of two nucleosides (spongethymide and spongouridine) from a marine sponge (Bergmann & Feeney, 1950, Bergmann & Feeney, 1951). From that, Ara-C (first marine sponge-derived anticancer agent) and Ara-A (an antiviral drug) were synthesized (Newman & Cragg, 2004). The bioactive compounds azidothymidine (AZT), used in HIV/AIDS treatment and acyclovir, an antiviral drug were obtained through modifications of the previous structures.

Over the last few decades hundreds of compounds were obtained from marine sponges and were shown to have a huge potential as drugs. There are more sponge-derived compounds in clinical and preclinical trials than any other marine phylum (Blunt et al., 2005). But, a supply issue raised, as these compounds are in very small amounts in sponges, and sponges have a low growth rate and difficult accessibility for isolation, hampering investigations for pre and clinical trials. One example is halichondrin B, first isolated from the marine sponge *Halichondria okadai*. This compound showed a high anticancer activity but, according to Munro et al. (1999) for initial clinical trials it was estimated the necessity of about 10g and 1 to 5 kg per year as a commercial drug. *Lissodendoryx* sp. showed to be the sponge producing higher quantities of halichondrin B, with about 300g/ton of sponge and an estimation of entire natural biomass of 289 tons. These numbers ruled out natural harvesting, and aquaculture showed to be economically untenable (Taylor et al., 2007a). Due to the structure complexity of this compound total synthesis showed also to be impractical (Taylor et al., 2007a). In 2005 a synthetic analogue, E7389 that retains the potency of the parent compound entered phase I clinical trials as an anticancer drug (Simmons et al., 2005). This compound is now approved by the FDA (U.S. Food and Drug Administration) and the European Medicines Agency for breast cancer and liposarcoma treatment.

Cyanobacteria are one of the oldest forms of life. During their evolution, production of secondary metabolites showed to be essential, allowing them to adapt to various environmental conditions such as higher temperature, pH variations, etc. Those secondary metabolites started being investigated because of their toxic effect. Due to eutrophication and climate changes, cyanobacterial blooms increased both in frequency and extension in the last decades in water bodies, posing health risks to populations and animals. But the potential use of them is much more extended and are already being used in agriculture industry (biocides, biofertilizers), cosmetics (UV radiation block), pharmaceutical industry (ant-HIV, antiviral, antitumor, antifungal, antiplasmodial, antibacterial, immunosuppressant, anticoagulant, anti-inflammatory, antiprotozoal, antituberculosis, etc.), and many other commercial uses (biofuel, bioremediators,

chelators, food supplements) (Haque et al., 2017, Swain et al., 2017). Many of these compounds were proved to be greener chemical compounds for a more sustainable future. Moreover, cyanobacteria are a source of peptides, trans-fatty acids, amino-acids, vitamins, carotenes, chlorophyll, phycocyanin and minerals (Mimouni et al., 2012), with compounds from different classes (peptides, alkaloids, terpenoids, macrolides, polyketides, fatty-acids, cyclophanes, etc.) (Swain et al., 2017). According to Gerwick and Moore (2012) it is likely that approximately 20% of small molecules with FDA approval and in clinical trials have cyanobacteria as predicted biosynthetic source. Apart from producing such a wide range of compounds, it is also known that cyanobacteria can affect the biosynthesis of compounds from marine invertebrates such as sponges (Ridley et al., 2005).

Most existing studies on the toxicological potential of cyanobacteria focuses on freshwater cyanobacteria, with less information on marine environments. The bioactive potential of both freshwater and marine cyanobacteria are known to be different (Swain et al., 2017). According to Mi et al. (2017), from 2007 to 2016, more than 400 new natural compounds were discovered from marine cyanobacteria. Coastal water blooms have also increased posing another concern, as cyanobacterial toxins are able to accumulate in both vertebrates and invertebrates (Buratti et al., 2017). In Portugal a huge effort is being made to address this issue as presented in the works of Brito et al. (2012), Leão et al. (2013), Costa et al. (2014), Brito et al. (2015), Costa et al. (2015). Ramos et al. (2018) made already a review of the potential chemodiversity of many cyanobacterial strains deposited in LEGE CC. Many of these strains were isolated from the coast of Portugal.

Sponges, as filter-feeders harbour a huge diversity of microorganisms such as cyanobacteria and are capable of concentrating some of them exceeding up to 4 orders of magnitude the microbial diversity in water column (Hentschel et al., 2006). Sponges can be used as a source for cyanobacteria harvesting. Some compounds, previously extracted from marine sponges were proven to be produced by symbiotic cyanobacteria. *Oscillatoria spongeliae* has been found to be the true source of some compounds isolated from marine sponges and with antibacterial and therapeutic properties (Unson & Faulkner, 1993, Unson et al., 1994, Thomas et al., 2010b). One example is the metabolite 2-(2',4'- dibromophenoxy)-4,6-dibromophenol. This compound, firstly extracted from the surface tissues of the marine sponge *Dysidea herbacea* was than only found in cells of the cyanobacteriam *Oscillatoria spongeliae* (Unson et al., 1994).

Thesis outline

The main objectives of the present work are stated bellow and are addressed in the present thesis as outlined, with each objective as a different chapter:

1. Study the diversity and distribution of sponges in the Portuguese coast (Chapter 2);
   There is a lack of information about marine sponges, especially intertidal species from the coast of Portugal. In the present study aimed to address this issue, using an integrative approach based the identification on both morphological, ecological and molecular parameters, and focusing on the western coast of Portugal intertidal area; Since most information from sponge diversity are present in master and PhD thesis, not available for most researchers (many of them prior to the 90's) a comprehensive listing on both diversity and location of sponge species was also made.

2. Assess the diversity of cyanobacteria associated with marine sponges using culture dependent and molecular approaches (Chapter 3);
   Not all microbial community from sponges can be cultured. Starting with this permise, we wanted to see which cyanobacteria we would be able to isolate and grow under laboratory conditions and then compare it with molecular identified cyanobacteria through DGGE and with their free-living counterparts. We aimed to investigate the diversity of cyanobacteria associated with the intertidal marine sponge host *Hymeniacidon perlevis*, collected along the coast of Portugal (Northeast Atlantic) and along a year and compare their DGGE fingerprint profiling.

3. Understand the diversity of microorganisms and especially of cyanobacteria within sponges and how laboratory maintenance of sponges can affect their microbial community (Chapter 4);
   Sponges, due to their phylogenetic position can become good animal models for several studies, and many compounds with pharmaceutical interest extracted from sponges are known to be produced by associated microorganisms but only when associated with the host. Also, sponges and their microbial community must be studied as one metaorganism. Studying how translocation of sponges from *in situ* conditions, to laboratory maintenance can affect their bacterial community is the first step towards understanding how well the community is maintained and how it affects sponge viability. The use of NGS techniques will help answer this question for the

intertidal marine sponge *H. perlevis*. The use of TEM analysis, combined with the NGS information will give insights on the cyanobacterial community, and its importance in the sponge.

4. Study the toxicological potential from cultured cyanobacteria isolated from marine sponges (Chapter 5);

   Co-evolution of sponges and cyanobacteria have already been documented with genome adaptations of the cyanobionts. Free living cyanobacteria have been the focus of many studies aiming to address secondary metabolite production as a source of novel natural compounds. Since there are adaptation of cyanobionts, the aim of the present chapter was to address the toxicological potential of cyanobacteria isolated from marine sponges through a series of ecologically-relevant bioassays.

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Chapter 2. Diversity of intertidal marine sponges from the western coast of Portugal (Northeast Atlantic)

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(Submitted manuscript to Journal of the Marine Biological Association of United Kingdom)

Appendix 1.

Literature review on Porifera diversity from the coast of Portugal. It is presented a table with a revision of sponge diversity from the coast of Portugal, already containing the information about sponge diversity obtained from the present study.

Appendix 2.

Here is presented a booklet, a brochure and a poster for scientific divulgation, made during this work with the most abundant demosponges in the northern intertidal coast of Portugal.

Diversity of intertidal marine sponges from the western coast of Portugal (Northeast Atlantic)

Abstract

Sponges are important components of the intertidal marine communities. There is a lack of information about intertidal marine sponge diversity in the western coast of Portugal (North-East Atlantic). This region has some particular biogeographic circumstances, with climatic influences from the Mediterranean Sea and the Atlantic Ocean. In the present work we identified the most common intertidal sponges of the western coast of Portugal and made a comprehensive list of the intertidal species described so far for this region. Sponges belonging to the Class Calcarea and Demospongiae were identified, the former class for the first time at these locations. Demospongiae are the most common intertidal sponges, present in all sampling locations. We used an integrative approach for Demospongiae identification, using both morphological and molecular characters. Molecular identification, using CO1 marker proved to be helpful in the identification to the genus level, despite some limitations, such as difficulty in amplification experienced for sponges as well as non-target organisms. The western coast of Portugal is shown to have a high diversity of intertidal sponge. The demosponge *Hymeniacidon perlevis* was present at all sample locations. Calcarean species were primarily found in samples taken along the southwestern coast.

Keywords

Porifera, Intertidal diversity, CO1, Portugal, Calcarea, Demospongiae

Introduction

Porifera is the oldest metazoan group still extant in our planet and one of the most abundant groups of animals. These organisms are key members of shallow- and deep-water benthic ecosystems, occupying all aquatic environments, from marine to freshwater, tropical, temperate and polar areas (Sarà & Vacelet, 1973, Van Soest et al., 2012). There are more than 8500 species (according to World Porifera Database (Van Soest et al., 2017)) of Porifera accepted and an additional 2300-3000 species already identified (Appeltans et al., 2012). The Class Demospongiae comprises 83% of all living sponges (Van Soest et al., 2012, Morrow & Cárdenas, 2015). Sponges play crucial steps

of the cycle of dissolved nutrients and organic matter in marine environments (Bell, 2008, Maldonado et al., 2012), and are a vast source of compounds with biotechnological applications (Leal et al., 2012).

Hooper and van Soest (2002) published a revised book on sponge classification improving our knowledge in sponge biodiversity. This classification relies greatly in spicules morphology and their arrangement in sponge tissue (Morrow et al., 2013). The problem with this classification is that sponges are invertebrates with a high degree of ecophenotypic plasticity, influenced by parameters such as light, sedimentation, substratum type and orientation, and water-flow regime, resulting in unresolved and ambiguous classification (Bell & Barnes, 2000, Erpenbeck et al., 2006, Van Soest et al., 2012, Erpenbeck et al., 2016). Also, many of these morphological characters can be non-homologous, resulting in unresolved and ambiguous classification (Boury-Esnault, 2006). Problems related to identification resulted in disregarding sponges in large-scale surveys. In order to overcome this issue, molecular characters are being used as an aid for resolving these limitations (Wörheide et al., 2005, Wörheide et al., 2007, Cárdenas et al., 2009, Cárdenas et al., 2010, Pöppe et al., 2010, Vargas et al., 2012, Boury-Esnault et al., 2013). Although phylogenetic studies have shown that the four Porifera classes are monophyletic, many major clades of sponges appear to be paraphyletic, leading to a revision of traditional sponge classification (Cárdenas et al., 2012, Hill et al., 2013, Thacker et al., 2013, Morrow & Cárdenas, 2015, Alvizu et al., 2018).

In sponge phylogenetic studies, many different molecular markers have been used, both nuclear and mitochondrial. A 5' partition of the mitochondrial cytochrome oxidase subunit 1 (CO1) (Folmer et al., 1994) is among the most popular markers, being used for the "barcoding of life" initiative. The Sponge Barcoding Project (Wörheide et al., 2007) was the first one on any non-bilateral taxon, aiming to cover all sponge taxa using primarily the 5' partition of CO1 marker.

The western coast of Portugal extends for more than 600 km and has some particular biogeographic circumstances (Boaventura et al., 2002), with climatic influences from the Atlantic Ocean and Mediterranean Sea (Kottek et al., 2006). As a result, biodiversity is a mixture of the one present in the North-western Atlantic coasts and the Mediterranean (Boaventura et al., 2002). Although sponges can be dominant members of some communities and play important roles in a variety of ecosystem functions (Rützler, 2012, Wulff, 2012), our knowledge of the intertidal and subtidal marine sponges in western Portugal derives especially from the works of Hanitsch (1895), Lévi and Vacelet (1958), Saldanha (1974), Lopes (1989), Pereira (2007). In recent years, due to difficulties in sponge identification, most intertidal diversity studies performed in this area (for example:

Monteiro Marques et al. (1982), Boaventura et al. (2002), Pereira et al. (2006)) neglected phylum Porifera and, improving our understanding of their biodiversity can be essential for habitats protection.

The aim of the present study is to characterize sponge diversity from the western coast of Portugal (NE Atlantic) using both morphological and molecular characters.

Materials and methods

Study site

Sampling took place between September 2010 and September 2014 in Portugal (North East Atlantic) and were made during the lowest tide hours of the month (below 0.5 m of mean sea level). All beaches had a combination of sand and rocks. Figure 2-1(a-c) show three different sampling locations. Collected sponges inhabit the rocky intertidal region and were predominant in sheltered areas, protected from the strong sun and tide, often lying at the base of the rocks.

Sponge samples were collected from 12 different intertidal sites, as it is shown in Figure 2-1. A total of 35 collection trips were made and 179 sponges sampled. Sponges were on rock overhangs, and through wading and the help of a knife they were collected. After collection, sponges were immediately carried to the laboratory and processing began within 1h after collection and to a maximum of 28 h.

Samples were photographed and preserved in 96% ethanol both for molecular analysis and morphological identification.

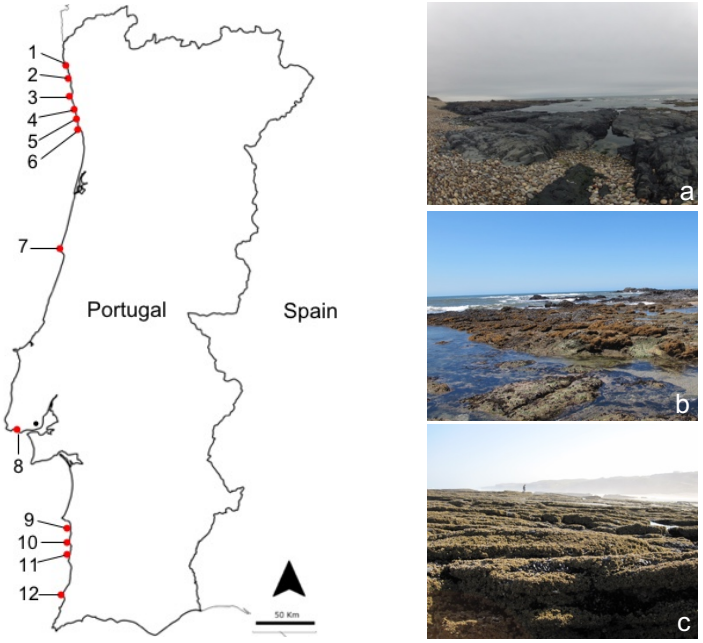

Figure 2-1. Sampling locations in Portugal: (1) Viana do Castelo (N 41º 41' 48,79" ,W 8º 51' 4,03"), (2) Esposende (N 41º 34' 25,59" ,W 8º 47' 54,81"), (3) Apúlia (N 41º 29' 17,34" ,W 8º 46' 59,38"), (4) Angeiras (N 41º 16' 6,08" ,W 8º 43' 33,39"), (5) Memória (N 41º 13' 52,27", W 8º 43' 18,34"), (6) Aguda (N 41º 2' 58,35'', W 8º 39' 19,22''), (7) Buarcos (N 40º 9' 22,36" ,W 8º 52' 18,49"), (8) S. João do Estoril (N 38º 41' 31,68" ,W 9º 21' 57,74"), (9) Porto Côvo (N 37º 52' 3,04'', W 8º 47' 37,19"), (10) Vila Nova de Milfontes (N 37º 42' 58,61" ,W 8º 47' 4,79"), (11) Almograve (N 37º 39' 2,7" ,W 8º 48' 10,8"), (12) Monte Clérigos (N 37º 20' 29,35" ,W 8º 51' 10,05"). Pictures (a), (b) and (c) ilustrate 3 of the sampling locations: Esposende (a), Memória (b) and Porto Côvo (c).

Sponge identification

Sponges were identified based on shape, consistency, texture, colour, habitat and spicules morphology, dimensions and arrangement. All sponge species collected were identified according to Hooper and van Soest (2002).

Molecular analyses

DNA extraction

Total genomic DNA was extracted from sponge tissue (choanossomal tissue) using a commercially available Purelink™ Genomic DNA mini Kit (Invitrogen, San Diego, CA) and stored at −20 °C until further analyses. gDNA integrity was checked by agarose gel electrophoresis with GelRed™ (Biotium) staining.

PCR and sequencing of cyanobacterial cultures

PCR amplification was done for a fragment located at the 5' site of the mitochondrial cytochrome oxidase subunit 1 (CO1). Primers used were designed by Meyer et al. (2005), based on the ones described by Folmer et al. (1994). PCR conditions employed were as follows: initial denaturation at 94 ºC for 2 min, followed by 35 cycles of denaturation at 94 ºC for 40 s, annealing at 50 ºC for 40 s and extension at 72 ºC for 1 min and a final extension step at 72 ºC for 10 min. When necessary, amplification was done using primer forward from Meyer et al. (2005), combined with the reverse from Xavier et al. (2010). This reverse primer amplify an alternative partition of the CO1 gene

that overlaps approximately 60 bp with Folmer's 3' partition and includes Erpenbeck's I3-M11 (Erpenbeck et al., 2006), a partition known to be more informative in cases of shorter divergence times. The incorporation of the primer designed by Xavier et al. (2010), showed to be more sponge specific, helping overcome problems related with amplification of non-target DNA. The following protocol was used: initial denaturation at 95 ºC for 3 min, followed by 35 cycles of denaturation at 94 ºC for 40 s, annealing at 54 ºC for 45 s and extension at 72 ºC for 90 s and a final extension step at 72 ºC for 10 min. A 5-10 ng of DNA were used for the PCR amplification. All PCR reactions were prepared in a 50 µL volume using Supreme NZYTaq 2x Green MasterMix (NZYTech, Lisboa, Portugal). Thermal cycling was carried out using Biometra T-Professional standard thermocycler (Biometra, Goettingen, Germany). PCR products were separated by 1.5% (w/v) agarose gel in 1x TAE buffer (Invitrogen, San Diego, CA, USA). The gels were stained with GelRed™ (Biotium, Fremont, CA, USA) and photographed under UV transillumination. For DNA sequencing each amplified product was purified using an Invitrogen PureLink™QuickGel Extraction and PCR Purification Combo Kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol followed by direct sequencing (GATC Biotech, Cologne, Germany).

Phylogenetic analysis

The sequences obtained were analysed using Geneious® v9.1.5 software (Kearse et al., 2012). The final sequences were used for similarity search using BLAST and the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequences were aligned with Muscle (Edgar, 2004). Ambiguously aligned positions and gaps were removed with GBlocks (Castresana, 2000) using less stringent parameters. Maximum-likelihood (ML) phylogenetic trees (Felsenstein, 1981a) were constructed in PhyML (Guindon & Gascuel, 2003b). MrBayes v3.2.5 (Huelsenbeck et al., 2001b) was used to perform a Bayesian inference (BI) analysis. The best fit evolutionary models- TrN+I+G and HKY+I+G under Akaike Information Criterion with correction (AICc) implemented in MrAIC v1.4.6 (Nylander, 2004) were selected for ML and BI respectively. As the point of the phylogenetic analysis was not to make any evolutionary inference, focusing on sponge diversity rather than evolutionary relationship, unrooted tree was used.

Nucleotide sequence accession number

All sequences were submitted to the GenBank database (accession numbers KY492518-KY492600).

Results

Sampling locations were selected along the entire western coast of Portugal (Figure 2-1). Only rocky shore locations were selected as sponges are sessile animals that settle on hard surfaces. Sampling periods were restricted to a few hours because of tidal regimes. To gain access to the largest possible intertidal area, sampling was always schedule during spring tide.

A total of 7 sponges (five species) were identified as belonging to the class Calcarea and 172 specimens (23 species) to the class Demospongiae. Among Demospongiae, all species identified belonged to the subclasses Heteroscleromorpha, Keratosa and Verongimorpha. Table 2-1 shows the species identification in accordance to location. Figure 2-2 shows pictures of the 30 identified sponge species. This identification is based on the morphological characters and, when obtained, confirmed by molecular analyses.

Table 2-1. Sponges collected from the western coast of Portugal. Sponges are divided in accordance to Class (Calcarea and Demospongiae) and their geographical locations are identified.

		North						Centre		South			
Class	Species	Viana do Castelo	Esposende	Apúlia	Angeiras	Memória	Aguda	Buarcos	S. João do Estoril	Porto Côvo	V N Mil Fontes	Almograve	Aljezur
Calcarea	*Grantia compressa*										X		X
	Leucandra gossei												X
	Sycon ciliatum										X		
	Clathrina coriacea					X					X		
	Clathrina blanca					X							
Demospongiae	*Stelligera rigida*					X							
	Cliona celata					X		X					
	Haliclona sp.					X							
	Haliclona (Rhizoniera) rosea							X					
	Haliclona (Haliclona) simulans	X				X	X	X					
	Crella (*Yvesia*) *rosea*					X							
	Amphilectus fucorum		X			X	X	X					
	Hymedesmia (*Hymedesmia*) *jecusculum*					X							
	Phorbas plumosus					X	X	X					
	Antho (*Antho*) *granditoxa*					X							
	Clathria (Clathria) coralloides	X				X							
	Ophlitaspongia papilla	X				X							X
	Myxilla (*Myxilla*) *rosacea*					X							
	Tedania (*Tedania*) *pilarriosae*					X							
	Polymastia sp.					X							
	Polymastia sp.					X							
	Polymastia agglutinans					X							

Polymastia penicillus					X						
Halichondria (*Halichondria*) *panicea*	X	X			X	X	X	X			
Hymeniacidon perlevis	X	X	X	X	X	X	X	X	X	X	X
Aaptos aaptos					X						
Aaptos papillata					X						
Dysidea fragilis					X						
Ircinia variabilis					X					X	
Aplysilla rosea						X					

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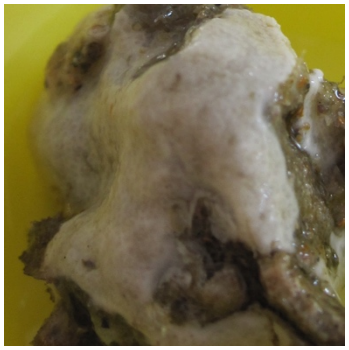

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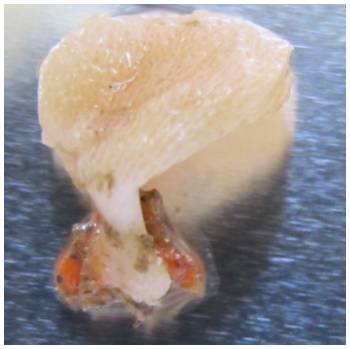

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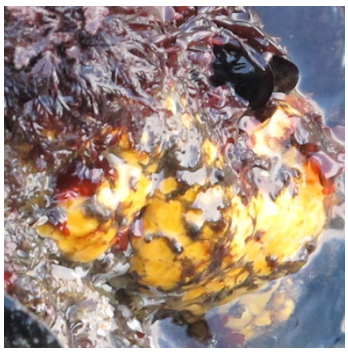

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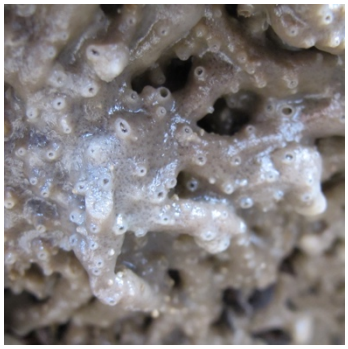

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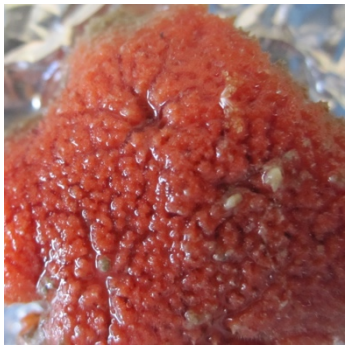

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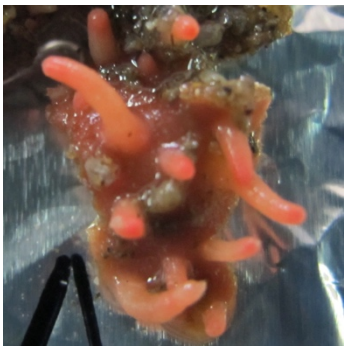

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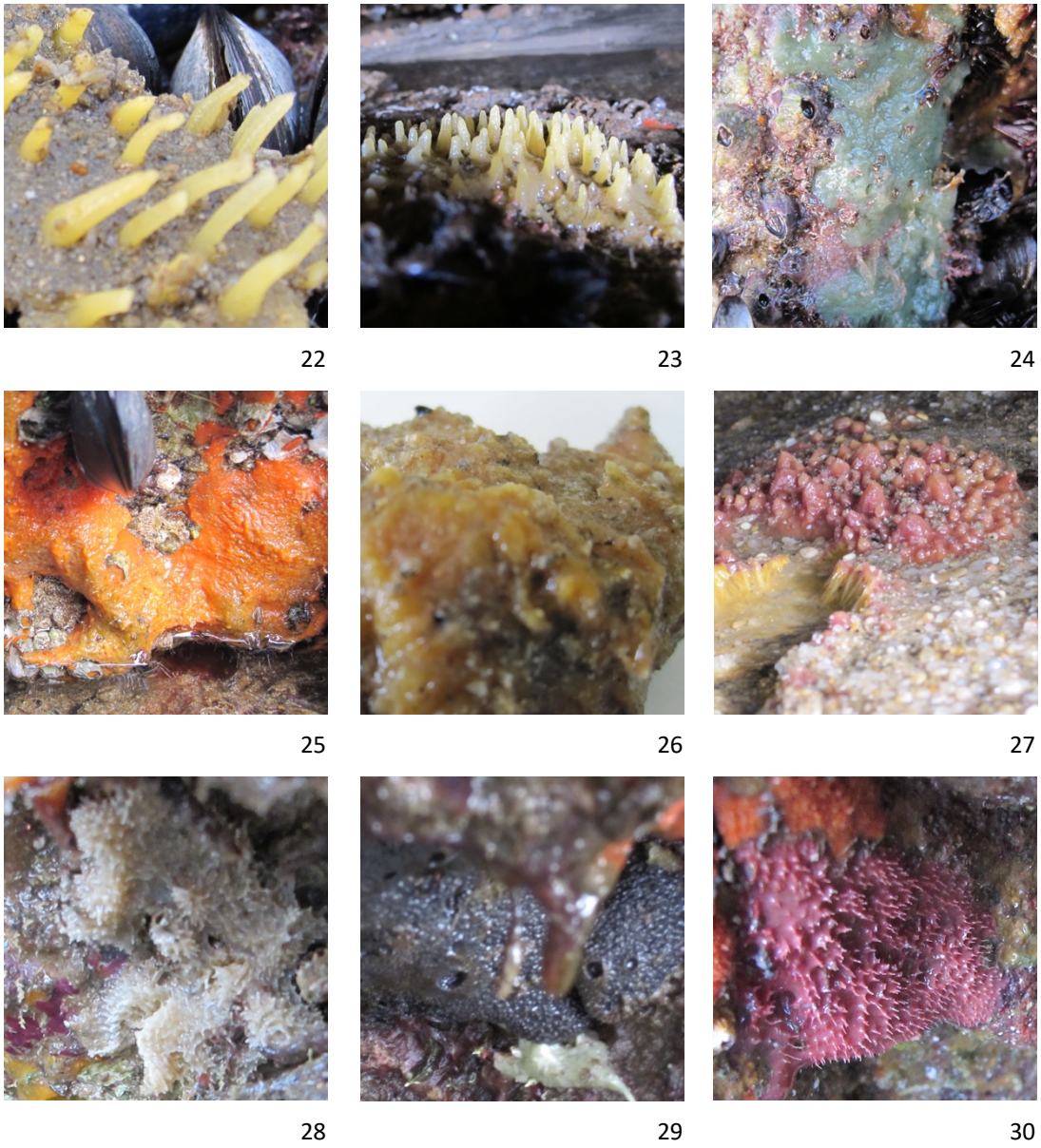

Figure 2-2. Pictures of identified sponges: 1. *Grantia compressa*, 2. *Leucandra gossei*, 3. *Sycon ciliatum*, 4. *Clathrina coriacea*, 5. *Clathrina blanca*, 6. *Stelligera rigida*, 7. *Cliona celata*, 8. *Haliclona* sp., 9. *Haliclona (Rhizoniera) rosea*, 10. *Haliclona (Haliclona) simulans*, 11. *Crella (Yvesia) rosea*, 12. *Amphilectus fucorum*, 13. *Hymedesmia (Hymedesmia) jecusculum*, 14. *Phorbas plumosus*, 15. *Antho (Antho) granditoxa*, 16. *Clathria (Clathria) coralloides*, 17. *Ophlitaspongia papilla*, 18. *Myxilla (Myxilla) rosacea*, 19. *Tedania (Tedania) pilarriosae*, 20. *Polymastia* sp., 21. *Polymastia* sp., 22. *Polymastia agglutinans*, 23. *Polymastia penicillus*, 24. *Halichondria (Halichondria) panicea*, 25. *Hymeniacidon perlevis*, 26. *Aaptos aaptos*, 27. *Aaptos papillata*, 28. *Dysidea fragilis*, 29. *Ircinia variabilis*, 30. *Aplysilla rosea*.

List of intertidal demosponges from the western coast of Portugal

Species with an asterisk (*) correspond to the ones found in the present work. After the name of the species, is given the reference for the first record for the western coast of Portugal.

Class CALCAREA Bowerbank, 1862

Subclass CALCARONEA Bidder, 1898
Order LEUCOSOLENIDA Hartman, 1958
Family GRANTIIDAE Dendy, 1893
Genus *Grantia* Fleming, 1828
**Grantia compressa* (Fabricius, 1780) (Pereira, 2007)

Genus *Leucandra* Haeckel, 1872
**Leucandra gossei* (Bowerbank, 1862) (Saldanha, 1974)

Family SYCETTIDAE Dendy, 1893
Genus *Sycon* Risso, 1827
**Sycon ciliatum* (Fabricius, 1780) (Saldanha, 1974)

Subclass CALCINEA Bidder, 1898
Order CLATHRINIDA Hartman, 1958
Family CLATHRINIDAE Minchin, 1900
Genus *Clathrina* Gray, 1867
**Clathrina coriacea* (Montagu,1814) (Hanitsch, 1895)
**Clathrina blanca* (Miklucho-Maclay, 1868) (Pereira, 2007)

Class DEMOSPONGIAE Sollas, 1885

Subclass HETEROSCLEROMORPHA Cárdenas, Pérez & Boury-Esnault, 2012
Order AXINELLIDA Lévi, 1953
Family RASPAILIIDAE Nardo, 1833
Genus *Eurypon* Gray, 1867
Eurypon clavatum (Bowerbank, 1866) (Lopes, 1989)
Eurypon coronula (Bowerbank, 1874) (Lopes, 1989)

Family STELLIGERIDAE Lendenfeld, 1898
Genus *Stelligera* Gray, 1867
**Stelligera* rigida (Montagu, 1814) (Lopes, 1989)

Order BUBARIDA Morrow & Cárdenas, 2015
Family DICTYONELLIDAE van Soest, Diaz & Pomponi, 1990
Genus *Tethyspira* Topsent, 1890
Tethyspira spinosa (Bowerbank, 1874) (Lopes, 1989)

Order CLIONAIDA Morrow & Cárdenas, 2015
Family CLIONAIDAE d'Orbigny, 1851
Genus *Cliona* Grant, 1826
**Cliona celata* Grant, 1826 (Saldanha, 1974)
Cliona viridis (Schmidt, 1862) (Saldanha, 1974)

Genus Pione Gray, 1867
Pione vastifica (Hancock, 1849) (Saldanha, 1974)

Order HAPLOSCLERIDA Topsent, 1928
Family CHALINIDAE Gray, 1867
Genus *Haliclona* Grant, 1841
**Haliclona* sp.
**Haliclona (Rhizoniera) rosea* (Bowerbank, 1866)
**Haliclona (Haliclona) simulans* (Johnston, 1842)

Order POECILOSCLERIDA Topsent, 1928
Family COELOSPHAERIDAE Dendy, 1922
Genus *Lissodendoryx* Topsent, 1892
Lissodendoryx (Lissodendoryx) isodictyalis (Carter, 1882) (Saldanha, 1974)

Family CRELLIDAE Dendy, 1922
Genus *Crella* Gray, 1867
**Crella (Yvesia) rosea* (Topsent, 1892)

Family ESPERIOPSIDAE Hentschel, 1923
Genus *Amphilectus* Vosmaer, 1880
**Amphilectus fucorum* (Esper, 1794) (Lopes, 1989)

Family HYMEDESMIIDAE Topsent, 1928
Genus *Hymedesmia* Bowerbank, 1864
**Hymedesmia (Hymedesmia) jecusculum* (Bowerbank, 1866)
Hymedesmia (Hymedesmia) pansa Bowerbank, 1882 (Lopes, 1989)
Hymedesmia (Stylopus) coriacea (Fristedt, 1885) (Lopes, 1989)

Genus *Phorbas* Duchassaing & Michelotti, 1864
Phorbas dives (Topsent, 1891) (Lopes, 1989)
Phorbas fictitious (Bowerbank, 1866) (Saldanha, 1974)

**Phorbas plumosus* (Montagu, 1814) (Lopes, 1989)

Family MICROCIONIDAE Carter, 1875

Genus *Antho* Gray, 1867

**Antho (Antho) granditoxa* Picton & Goodwin, 2007

Antho (Antho) involvens (Schmidt, 1864) (Lopes, 1989)

Genus Clathria Schmidt, 1862

**Clathria (Clathria) coralloides* (Scopoli, 1772) (Lopes, 1989)

Clathria (Clathria) toxistricta Topsent, 1925 (Pereira, 2007)

Clathria (Microciona) atrasanguinea (Bowerbank, 1862) (Lopes, 1989)

Clathria (Microciona) strepsitoxa (Hope, 1889) (Lopes, 1989)

Genus *Ophlitaspongia* Bowerbank, 1866

**Ophlitaspongia papilla* Bowerbank, 1866 (Costa, 2012)

Family MYCALIDAE Lundbeck, 1905

Genus *Mycale* Gray, 1867

Mycale (Aegogropila) contarenii (Lieberkühn, 1859) (Lopes, 1989)

Mycale (Carmia) macilenta (Bowerbank, 1866) (Lopes, 1989)

Mycale (Carmia) minima (Waller, 1880) (Lopes, 1989)

Family MYXILLIDAE Dendy, 1922

Genus *Myxilla* Schmidt, 1862

**Myxilla (Myxilla) rosacea* (Lieberkühn, 1859) (Hanitsch, 1895)

Family TEDANIIDAE Ridley & Dendy, 1886

Genus *Tedania* Gray, 1867

Tedania (Tedania) anhelans (Vio in Olivi, 1792) (Saldanha, 1974)

**Tedania (Tedania) pilarriosae* Cristobo, 2002

Order POLYMASTIIDA Morrow & Cárdenas, 2015

Family POLYMASTIIDAE Gray, 1867

Genus *Polymastia* Bowerbank, 1862

**Polymastia* sp.

**Polymastia* sp.

**Polymastia agglutinans* Ridley & Dendy, 1886

**Polymastia penicillus* (Montagu, 1814) (Saldanha, 1974)

Order SUBERITIDA Chombard & Boury-Esnault, 1999
Family HALICHONDRIIDAE Gray, 1867
Genus Halichondria Fleming, 1828
**Halichondria (Halichondria) panicea* (Pallas, 1766) (Carter, 1876)

Genus *Hymeniacidon* Bowerbank, 1858
**Hymeniacidon perlevis* (Montagu, 1814) (Hanitsch, 1895)

Family SUBERITIDAE Schmidt, 1870
Genus *Aaptos* Gray, 1867
**Aaptos aaptos* (Schmidt, 1864)
**Aaptos papillata* (Keller, 1880) (Lopes, 1989)

Genus *Protosuberites* Swartschewsky, 1905
Protosuberites epithyum (Lamark, 1815) (Lopes, 1989)

Genus *Pseudosuberites* Topsent, 1896
Pseudosuberites mollis Topsent, 1925 (Lopes, 1989)

Genus *Suberites* Nardo, 1833
Suberites carnosus (Johnston, 1842) (Lopes, 1989)

Genus *Terpios* Duchassaing & Michelotti, 1864
Terpios fugax Duchassaing & Michelotti, 1864 (Lopes, 1989)

Order TETHYIDA Morrow & Cárdenas, 2015
Family HEMIASTERELLIDAE Lendenfeld, 1889
Genus *Adreus* Gray, 1867
Adreus fascicularis (Bowerbank, 1866) (Lopes, 1989)

Family TETHYIDAE Gray, 1848
Genus *Tethya* Lamark, 1815
Tethya aurantium (Pallas, 1766) (Hanitsch, 1895)

Family TIMEIDAE Topsent, 1928
Genus *Timea* Gray, 1867
Timea mixta (Topsent, 1896) (Lopes, 1989)

Order TETRACTINELLIDA Marshall, 1876

Family ANCORINIDAE Schmidt, 1870
Genus *Stelleta* Schmidt, 1862
Stelletta anancora (Sollas, 1886) (Lopes, 1989)
Stelletta hispida (Buccich, 1886) (Saldanha, 1974)

Family GEODIIDAE Gray, 1867
Genus *Erylus* Gray, 1867
Erylus discophorus (Schmidt, 1862) (Saldanha, 1974)

Genus *Geodia* Lamark, 1817
Geodia cydonium (Linnaeus, 1767) (Saldanha, 1974)

Order TRACHYCLADIDA Morrow & Cárdenas, 2015
Family TRACHYCLADIDAE Hallmann, 1917
Genus *Trachycladus* Carter, 1879
Trachycladus minax Topsent, 1888 (Lopes, 1989)

Subclass KERATOSA Grant, 1861
Order DICTYOCETARIDA Minchin, 1900
Family DYSIDEIDAE Gray, 1867
Genus *Dysidea* Johnston, 1842
**Dysidea fragilis* (Montagu, 1814) (Pérès, 1959)

Family IRCINIIDAE Gray, 1867
Genus *Ircinia* Nardo, 1833
**Ircinia variabilis* (Schmidt, 1862) (Hanitsch, 1895)

Genus *Sarcotragus* Schmidt, 1862
Sarcotragus spinosulus Schmidt, 1862 (Lopes & Boury-Esnault, 1981)
Sarcotragus fasciculatus (Pallas, 1766) (Saldanha, 1974)

Family SPONGIIDAE Gray, 1867
Genus *Spongia* Linnaeus, 1759
Spongia (Spongia) officinalis Linnaeus, 1759 (Lopes & Boury-Esnault, 1981)

Family THORECTIDAE Bergquist, 1978
Genus *Scalarispongia* Cook & Bergquist, 2000
Scalarispongia scalaris (Schmidt, 1862) (Lopes & Boury-Esnault, 1981)

Order DENDROCETARIDA Minchin, 1900
Family DARWINELLIDAE Merejkowsky, 1879
Genus *Aplysilla* Schulze, 1878
**Aplysilla rosea* (Barrois, 1876) (Lopes, 1989)

Subclass VERONGIMORPHA Erpenbeck, Sutcliffe, De Cook, Dietzel, Maldonado, van Soest, Hooper & Wörheide, 2012
Order CHONDRILLIDA Redmond, Morrow, Thacker, Diaz, Boury-Esnault, Cardenas, Hajdu, Lobo-Hajdu, Picton, Pomponi, Kayal & Colins, 2013
Family CHONDRILLIDAE Gray, 1872
Genus *Thymosia* Topsent, 1895
Thymosia guernei Topsent, 1895 (Lopes, 1989)

Order VERONGIIDA Bergquist, 1978
Family APLYSINIDAE Carter, 1875
Genus *Aplysina* Nardo, 1834
Aplysina aerophoba (Nardo, 1833) (Lopes, 1989)

For the 172 Demosponges collected, we were only able to retrieve DNA from 154. For that, we only recover sponge DNA for 85 of them. For the remain, obtained DNA had poor quality or amplified DNA from other small invertebrates or marine algae, and were discarded. The molecular analysis was made to apply an integrative taxonomy approach, complementing the morphological identification with molecular data and to assess the relative positioning of the identified Demospongiae. The phylogenetic tree (Figure 2-3) revealed a well-supported topology, both by Maximum Likelihood and Bayesian tree-reconstruction approach, clearly separating different sponge genera. All sequences obtained belong to the subclass Heteroscleromorpha and there is a clear distinction between the different orders. Specimens from the genus *Hymeniacidon, Halichondria* and *Aaptos* clustered together as all belong to the order Suberitida. In this clade is also possible to distinguish between different families (*Hymeniacidon* and *Halichondria* belong to the family Halichondriidae and *Aaptos* belong to the family Suberitidae) and different genera. Also, the genera *Tedania*, *Hymedesmia*, *Myxilla*, *Phorbas*, *Antho*, *Ophlitaspongia* and *Amphilectus* belong all to the order Poecilosclerida and are all clustered together. For almost all genera from this order, is also possible to distinguish between different families.

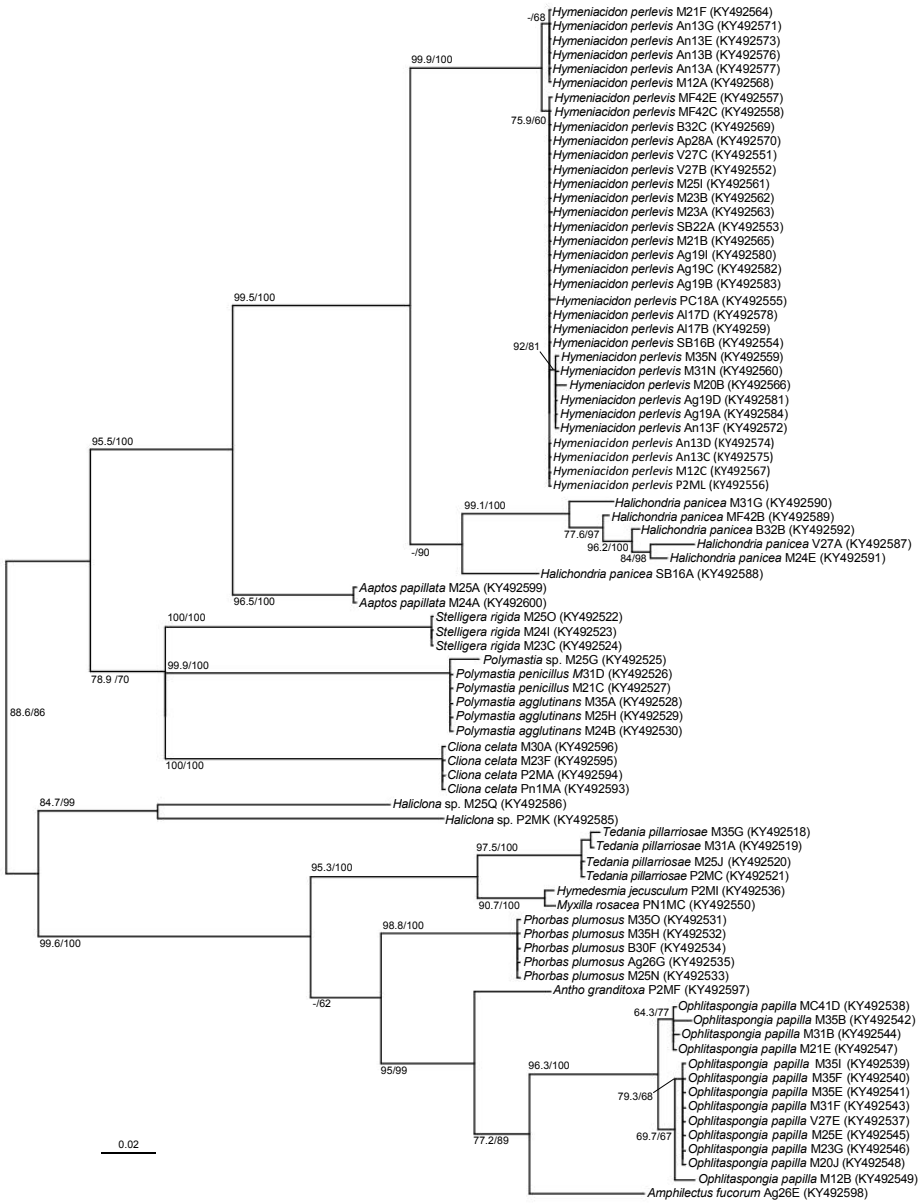

Figure 2-3. Maximum likelihood (ML) phylogenetic tree based on the CO1 fragment of the sequences from Demospongiae. GenBank accession numbers are given in parentheses. The tree is unrooted. Bayesian posterior probabilities and ML bootstrap support values are represented at the nodes. Only bootstrap values greater than 50% are given. The scale bar at the bottom represents 2% sequence divergence.

Discussion

The present study shows for the first time an updated list of intertidal sponges from the western coast of Portugal. Identified sponges belong to the class Calcarea (5 species), and Demospongiae. Praia da Memória, in the northern part of Portugal seems to harbour the higher diversity of demosponges. Sponges belonging to the class Calcarea are more dominant on the southern intertidal area of Portugal. The present work was the first focusing on calcarean sponges from the intertidal areas in this geographical location. So

far, there was no information for intertidal diversity of calcarean sponges (Hanitsch, 1895, Saldanha, 1974, Lopes, 1989, Pereira, 2007). From the 27 species of Demospongiae, 12 are described for the first time in the intertidal area and 11 for the first time on the western coast of Portugal. These results show the high diversity of sponges inhabiting the intertidal western coast of Portugal.

Most sponge diversity studies focus on underwater sponges (Carter, 1876, Topsent, 1928, Lévi & Vacelet, 1958, Saldanha, 1974, Lopes & Boury-Esnault, 1981, Naveiro, 2002, Pereira, 2007, Pires, 2007) and most intertidal diversity studies from this geographical area completely neglect the existence of sponges. Although lacking many typical characteristics of animals, genetically, sponges have many key metazoan gene families. This enable them to give insights on the evolution of metazoans, as all these phyla derive from a common ancestor (Müller et al., 2004). In Atlantic shores, sponges have been recognized as important members of the ecosystem, both in terms of biomass and species richness, playing significant roles in ecosystem functioning (Xavier & van Soest, 2012) due to being filter feeders. Economically they are also of major importance due to the vast production of secondary metabolites, either by their own chemistry or that of their symbionts. Cyanobacteria, a common sponge symbiont, and known for their active secondary metabolism, have already been reported in intertidal sponges from this geographical location (Alex et al., 2012, Alex et al., 2013, Alex & Antunes, 2015, Regueiras et al., 2017). New secondary metabolites from Porifera, all from Demospongiae, are among the most promising to use for pharmaceutical applications (Leal et al., 2012). Intertidal sponges can also be used as bioindicators for water quality monitoring. Mahaut et al. (2013) used *Hymeniacidon perlevis* as a bioindicator and reported it to have a higher accumulation capacity of contaminants than the mussel *Mytilus edulis* Linnaeus. As this sponge inhabits almost all western coast of Portugal, it can be used for water pollution studies in the future. These findings show the importance of the study of sponges, and knowing their diversity is the first step for every other study. Plasticity in sponge morphology is very common, which makes sponge identification a challenge. Barnes and Bell (2002) found differences in sponge morphology within the same species with varying depth.

To overcome this issue, many studies have been focusing on molecular data. CO1 has been the most popular marker, as it can help in taxonomy (Pöppe et al., 2010). Also, as it has been the marker chosen for the barcoding of life and the sponge barcoding project, and there is more information on public databases for this marker than for any other.

In our study, the use of CO1 helped to distinguish most of our sponges at the genus level. According to Cárdenas et al. (2012), CO1 is not the ideal sponge barcoding

marker, as it does not allow to distinguish between different species due to the slow evolutionary rate (Erpenbeck et al., 2016) and by the difficulty to sequence it. As here demonstrated, CO1 was previously shown to have a good resolution at the family level (Erpenbeck et al., 2002, Erpenbeck et al., 2016) and in some cases to the genus level (Erpenbeck et al., 2006).

We were not able to retrieve DNA for all Demospongiae. Extracting DNA from sponge tissue can have its challenges, as it is known that some taxa required specialized protocols (Erpenbeck et al., 2016) and some compounds can be present that can inhibit PCR reaction (Vargas et al., 2012). Also, the use of CO1 can result in co-amplification and/or specific amplification of non-target organisms (Vargas et al., 2012). According to Vargas et al. (2012) some Porifera families tend to be easier to amplify DNA than others. 55% of our samples showed poor DNA quality and/or amplification of DNA from non-target organisms. Vargas et al. (2012) found amplification of non-target organisms to happen in 40% of samples.

Erpenbeck et al. (2006) suggested the use of a downstream of the 5'-Folmer partition, which has a higher substitution rate to help distinguish between species or populations (Xavier et al., 2010). In order to overcome these problems, when necessary, we used a more specific primer, designed by Xavier et al. (2010). This approach allowed us to obtain more sequences but not for all Demospongiae. We only amplified this second region when we were not able to obtain target DNA, as this primer showed to be more sponge specific than the Folmer's one. In the future, it would be interesting to amplify all collected sponges using this partition, to help distinguishing phylogenetically between species and to see if its resolution can separate different populations of the same species in accordance with geographical distribution.

In this study, we presented for the first time a list of intertidal sponges from the western coast of Portugal, based on collection and identification and bibliography data. We presented also the first intertidal data for Calcarea intertidal sponges for the western coast of Portugal. We also showed advantages and limitations of using CO1 DNA data to help in the identification of Demospongiae. It seems that this marker is suitable for identification, in most cases, to the genus level but, to help distinguish species, another marker should be also used. A more specific primer for CO1 should also be used to decrease non-target DNA amplification. Also, a protocol for Demospongiae DNA extraction most be developed to overcome problems caused by contaminants that can inhibit PCR reaction.

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Chapter 3. Cyanobacterial diversity in the marine sponge *Hymeniacidon perlevis* from a temperate region (Portuguese coast, Northeast Atlantic)

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Cyanobacterial diversity in the marine sponge *Hymeniacidon perlevis* from a temperate region (Portuguese coast, Northeast Atlantic)

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Cyanobacterial diversity in the marine sponge *Hymeniacidon perlevis* from a temperate region (Portuguese coast, Northeast Atlantic)

Abstract

Cyanobacteria are commonly associated with marine sponges and are known to be difficult to isolate. In the present study, we used isolation and molecular techniques to investigate the diversity of *Cyanobacteria* associated with the intertidal marine sponge host *Hymeniacidon perlevis*, collected along the coast of Portugal (Northeast Atlantic). Cyanobacterial community profiling and comparison using 16S rRNA gene-sequence based denaturing gradient gel electrophoresis (DGGE) revealed different banding patterns between the sponge tissue and seawater. We succeeded in isolating *Cyanobacteria* belonging to the genera *Synechococcus*, *Cyanobium*, *Synechocystis*, *Nodosilinea*, *Pseudanabaena* and *Phormidesmis* from the sponge tissues. Chlorophyll a concentrations were very low, in spite of the diversity of cyanobacteria identified. DGGE analyses comparing sponge samples and ambient seawater further revealed the presence of *Synechococcus*, *Acaryochloris* and *Prochlorococcus*. Many of the isolated cyanobacteria show a high similarity with previously isolated free-living cyanobacteria from the coast of Portugal, highlighting the advantages of using sponges as a source for obtaining cyanobacteria present only in small amount in seawater.

Keywords

Cyanobacteria, Marine sponges, Diversity, Phylogeny, DGGE, North-eastern Atlantic coast

Introduction

Sponges are the most primitive multi-celled animals, with fossil records dating back 700 to 800 million years (Belarbi, 2003). They are known for harbouring a diversity of symbiotic microorganisms such as bacteria, fungi, unicellular algae and cyanobacteria (Taylor et al., 2007a). Based on the abundance and diversity of the microbial community they contain, sponges are classified as being high microbial abundance (HMA) or low

microbial abundance (LMA) sponges (Hentschel et al., 2003, Weisz et al., 2007). HMA sponges can contain a concentration of microorganisms 2 to 4 orders of magnitude higher than seawater (Friedrich et al., 2001, Hentschel et al., 2006). LMA sponges are typically smaller (Hentschel et al., 2006), with a smaller mesohyl and simpler aquiferous system but a higher pumping rate (Weisz et al., 2008).

Cyanobacteria, common photosymbionts, form associations with a wide variety of organisms in different habitats. In the marine environment, they are known to occur with sponges, ascidians, Echuroid worms, diatoms, dinoflagellates and protozoans (Carpenter & Foster, 2002). *Cyanobacteria* are an important group among the photosynthetic symbionts of sponges. Sponges with photosynthetic symbionts can constitute up to 85% of the total intertidal sponge communities in tropical reefs (Steindler et al., 2002) and up to 64% in temperate waters (Lemloh et al., 2009). According to Rützler (1990), unicellular and filamentous cyanobacteria can comprise up to 50% of a sponge's cellular volume. Cyanobacteria contribute to the relationship by transferring nutrients to the sponge, such as glycerol (Wilkinson & Fay, 1979), organic phosphate and nitrogen (Wilkinson & Fay, 1979), which enhances its growth rate and competiveness with other benthic communities (Wilkinson, 1980, Arillo et al., 1993). Cyanobacteria also provides UV protection as well as chemical defence through the production of secondary metabolites, as reviewed in Taylor et al. (2007a), Usher (2008), and Webster and Taylor (2012). Cyanobacteria can also benefit from the association with sponges, although the mechanisms are not as clear. The host provides shelter (Erwin & Thacker, 2007), and higher levels of ammonium and phosphorus than those present in the ocean (Usher, 2008). Primary productivity and nutrient cycling in marine ecosystems can also be enhanced by these symbioses (Diaz & Rützler, 2001). Vertical transmission of cyanobacterial symbionts (cyanobionts) to new generations has already been reported (Usher et al., 2001, Oren et al., 2005), which is considered to benefit the offspring by giving them photosynthetic energy before they are able to feed (Lemloh et al., 2009), enhancing their competitive fitness (Oren et al., 2005). Maldonado (2007) reported that in some sponges the symbiont is not transmitted to gametes or embryos, but instead they are obtained in each new generation from the environment (i.e. horizontal transmission). According to Schmitt et al. (2007) embryos from LMA sponges are typically microbe-free.

Cyanobacterial associations occur within the sponge classes Calcarea and Demospongiae (Carpenter & Foster, 2002). The sponge-associated *Cyanobacteria* identified so far belong to *Aphanocapsa*, *Synechococcus*, *Prochloron*, *Synechocystis*

and *Oscillatoria*. Recently, Alex et al. (2012) also reported the presence of *Xenococcus*-like and *Acaryochloris* sp. from the intertidal marine sponge *Hymeniacidon perlevis*. Some unknown species have also been found, as reviewed by Usher (2008). Some of these associations can occur in geographically distinct areas, and it is known that different sponges can have the same symbiont and each one can harbour more than one cyanobacterial species (Usher et al., 2006).

Cyanobacterial diversity in marine sponges has been the focus of many studies, mainly in tropical environments. Approximately 99% of the sponge associated microorganisms cannot be cultured (Santavy & Colwell, 1990, Friedrich et al., 2001, Hentschel et al., 2003, Isaacs et al., 2009) and, allied to the fact that the morphological characteristics are not enough to distinguish the cyanobacterial species (Usher et al., 2006), it is thought that the diversity is being underestimated and many relationships are yet to be discovered. In the last few years, molecular approaches have demonstrated that symbiotic cyanobacteria in sponges differ from those in the seawater communities (Usher et al., 2004, Steindler et al., 2005, Lemloh et al., 2009). These techniques have been able to assess the cyanobacterial diversity among the sponge hosts (Taylor et al., 2007a). Denaturing gradient gel electrophoresis (DGGE) has been commonly used to assess the diversity of *Bacteria* associated with marine sponges (Usher et al., 2004, Li et al., 2006, Wichels et al., 2006, Thiel et al., 2007, Lemloh et al., 2009, Anderson et al., 2010, Gerçe et al., 2011) and can provide insights into enrichment of the communities (Hentschel et al., 2003).

The aim of the present study was to assess the diversity of the cyanobacterial community in the most common intertidal LMA marine sponge, *Hymeniacidon perlevis* (Demospongiae, Halichondrida), distributed along the western coast of Portugal (NE Atlantic), using culture-based and molecular-based techniques. We also compared the phylogenetic relationships of the cyanobacterial community retrieved from sponge tissues and the water column with other, previously reported sponge-associated and free-living cyanobacteria, and discuss the ecological relevance of this study.

Materials and methods

Sample collection and preparation

Sampling was performed from September 2010 to September 2011 in Portugal (Northeast Atlantic). Specimens of the sponge *Hymeniacidon perlevis* were collected along the western coast of Portugal, during the lowest tide over each month (1.5 to 1.9 m below mean sea level). All selected sampling sites were beaches consisting of a

combination of sand and rocks. Sample collection only required a small portion of the sponge, which did not affect the animals' survival in the natural environment. For molecular purposes, only *H. perlevis* from 3 sampling locations (Memória, Aguda and Porto Côvo) (Figure 3-1) were used.

Samples were cleaned of debris and sediment, and placed in sterile 100 mL flasks containing filtered natural seawater from the sampling location. Water samples (150 mL) were also collected from each sampling location to isolate free-living cyanobacteria and for molecular-based analysis. After collection, sponge samples were immediately transported to the laboratory in a cooler on ice. Processing began between 1 and 6 h after sample collection. Samples were divided into 3 parts: one was processed immediately for the isolation of *Cyanobacteria*; one was preserved in 100% ethanol for subsequent genetic analysis; and one was preserved in 70% ethanol for morphological identification. For seawater, 150 mL samples were filtered through a 0.45 µm sterile filter followed by DNA extraction.

Sponges were identified based on the sampling habitat, shape, consistency, texture, colour, smell of the sponge sample and characteristic features (morphology, dimensions) of spicules. All sponge species were confirmed according to Hooper and van Soest (2002).

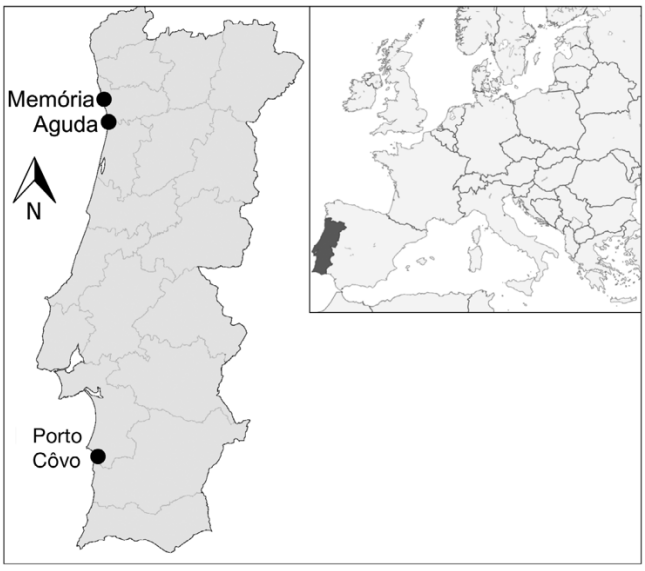

Figure 3-1. Sampling locations in Portugal (SW Europe) for denaturing gradient gel electrophoresis (DGGE) analysis: Memória (41° 13' 52.27''N, 8° 43' 18.34''W); Aguda (41° 2' 58.35''N, 8° 39' 19.22''W); and Porto Côvo (37° 52' 3.04''N, 8° 47' 37.19''W)

Chlorophyll *a* quantification

We employed the protocol described by Thacker (2005) to extract chlorophyll *a* (chl *a*) from marine sponges, assuming that most of the sponges harbour chl a-containing

cyanobacteria. Quantification of chl *a* was done in 9 specimens of *H. perlevis* collected from the sampling site at Memória. To summarize, 0.25 g from each sponge (wet weight) was extracted in 10 ml of 90% acetone, and kept overnight at 4°C. Three aliquots from the supernatant were used to determine absorbance at 630, 647, 664 and 750 nm. Chl *a* concentration was calculated using the equations of Parsons et al. (1984) standardized by sponge mass extracted.

Cyanobacteria culture and morphological characterization

To avoid the culturing of superficial bacteria, 1 mm of the exposed sponge surface tissues was removed with a sterile, double-sided razor. The remaining sponge samples were rinsed with distilled water to remove the transient and loosely attached organisms. Sections of the sponge body were used for culturing cyanobacteria. Small fragments (<0.5 cm^3) of sponge tissue were placed in 2 different media: Z8 liquid medium (Kótai, 1972) supplemented with 30 g l^{-1} of NaCl, and MN liquid medium (Rippka, 1988). The media were supplemented with vitamin B12 and cycloheximide (Rippka, 1988). The cultures were kept under 14 h light (10 to 30 µmol photons $m^{-2}s^{-1}$), 10 h dark cycles at 25°C. When cyanobacterial growth in the liquid was visible, an isolation procedure was done using a micromanipulation technique (Rippka, 1988), using a sterile *Pasteur* pipette to transfer a single cell or filament to liquid medium. Cyanobacteria cultures were achieved after several subcultures, and were unicyanobacterial and non-axenic. Water samples were centrifuged at 16 000 x *g* for 5 min (nSorval Legend RT centrifuge) and the pellet was placed in cyanobacterial culture media and kept under the same conditions as mentioned above.

Morphological cyanobacterial identification was performed following the criteria of Komárek and Anagnostidis (Komárek & Anagnostidis, 1998, Komárek & Anagnostidis, 2005, Komárek, 2013), using Bergey's manual of systematic bacteriology (Castenholz et al., 2001) and Komárek et al. (2014). Pictures were taken using an Olympus BX41 microscope (Olympus Europe) and analysed using Cell^B (Olympus Europe). Cyanobacterial isolates were deposited at LEGE Culture Collection (Laboratory of Ecotoxicology, Genomics and Evolution, CIIMAR, Porto, Portugal).

Molecular analyses

DNA extraction

Total genomic DNA (gDNA) was extracted from pure cyanobacterial cultures and sponge tissue, using a commercially available Purelink^{TM} genomic DNA mini kit (Invitrogen) following the protocol described for Gram-negative bacteria in accordance with the manufacturer's recommendations, and stored at -20 °C until further analysis. For the

water samples, 150 mL was centrifuged at 16 000 x *g* for 8 min followed by DNA extraction from the 'pellet' as described above. gDNA integrity was checked by agarose gel electrophoresis with ethidium bromide staining.

PCR and sequencing of cyanobacterial cultures

Two sets of primers were used for amplification and sequencing of 2 fragments of the partial 16S ribosomal RNA (rRNA) gene sequence, as shown in Table 3-1. PCR conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min. A total of 5 to 10 ng of DNA were used for the PCR amplification. All PCR reactions were prepared in a 50 µL volume containing 1x PCR buffer, 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate, 10 pmol of each primer, and 0.5 U of *Taq* DNA polymerase (Bioline). Thermal cycling was carried out using Biometra T-professional standard thermocycler (Biometra). PCR products were separated by 1.5% (w/v) agarose gel in 1x TAE buffer (Invitrogen). The gels were stained with ethidium bromide and photographed under UV transillumination. For DNA sequencing, each amplified product was purified using an Invitrogen PureLink™QuickGel Extraction and PCR Purification Combo Kit (Invitrogen) according to the manufacturer's protocol, followed by direct sequencing (Macrogen Europe). Sequences were deposited in GenBank database (accession numbers JQ927344, JQ927345, JQ927348, JQ927353 and KX608887 to KX608890).

Table 3-1. Primer pairs used in this study. F: Forward; R: Reverse

Target gene	Primer pair	Sequence (5′ to 3′ determination)	Size (bp)	Reference
16S rRNA	CYA106F	CGG ACG GGT GAG TAA CGC GTG A	675	Nübel et al. (1997)
	CYA781R (A)[a]	GAC TAC TGG GGT ATC TAA TCC CAT T		
	CYA781R (B)[a]	GAC TAC AGG GGT ATC TAA TCC CTT T		
	CYA359F	GGG GAA TYT TCC GCA ATG GG	1135	
	1494R	TAC GGC TAC CTT GTT ACG AC		Neilan et al. (1997)

[a](A) and (B): primers used together in a mixture with equimolar concentration

Screening of cyanobacterial community from sponge tissue and water samples

For 16S rRNA gene amplicons, a first round of PCR employing the *cyanobacteria*-specific primers CYA106F and CYA781R (described in Table 3-1) (Nübel et al., 1997) was followed by nested PCR reaction with GC-clamped primers, to amplify a *cyanobacteria* specific fragment from the 16S rRNA gene (16SCYA) with 359F-GC and 781R primers (Nübel et al., 1997). PCR conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 12 cycles of denaturation at 94 °C for 1 min, annealing at

65 °C for 1 min and extension at 72 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and a final extension step at 72 °C for 4 min.

A total of 20 µL of the PCR products corresponding to the 16S-CYA fragments were loaded into 6% polyacrylamide 1 mm gels, using a 30 to 55% denaturing gradient (100% denaturing conditions correspond to 7 M urea and 40% formamide). One gel was used to accommodate the 8 samples (4 specimens of *H. perlevis* and 4 seawater samples). Electrophoresis was performed using 1% TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA), at 60 V for 16 h, in a DCode system (Bio-Rad). The gels were stained with 1x SYBR Gold nucleic acid stain (Invitrogen) and selected DGGE bands were excised using a razor blade and placed in sterile microcentrifuge tubes with 10 µL of sterile Milli-Q H_2O. When bands with the same length appeared in different samples, only one of them was extracted; it was assumed that bands of the same length corresponded to the same cyanobacterial species. A total of 5 µL was used as a template in a new PCR reaction. This re-amplification was performed under the same conditions described in the previous section (see Table 3-1) for the corresponding fragment type, except that forward primers did not contain a GC-clamp. PCR products were excised from agarose gel and cleaned (Cut&Spin Gel Extraction columns, GRiSP) prior to cloning.

Purified PCR products from DGGE bands were then cloned into pGEM®-T Easy vector (Promega), and transformed into OneShot® TOP10 chemically competent *E. coli* cells (Invitrogen) using standard procedures (Sambrook & Russell 2001) and following the manufacturer's instructions. Plasmid DNA was isolated using GenElute™ plasmid miniprep kit (Sigma-Aldrich) and sequenced (Macrogen Europe) using M13 primers. For sequencing, 2 clones for each DGGE band were selected. Sequences obtained from DGGE clones were deposited in GenBank (accession numbers KC896629 to KC896638).

Phylogenetic analysis

The partial 16S rRNA gene sequences obtained were analysed using Geneious® v.9.1.5 software (www.geneious.com; Kearse et al. (2012)). The final sequence length, ranging from 345 to 1373 bp, was used for a similarity search using BLAST and the NCBI nucleotide database (www.ncbi.nlm.nih. gov/BLAST). A chimera check for derived 16S rRNA sequences was performed using Mallard (Ashelford et al., 2006). The sequences used in phylogenetic analyses were chosen to include (1) representatives of cyanobacterium diversity (reference strains), (2) sponge-associated cyanobacteria sequences overlapping with the new 16S rRNA sequences, and (3) representatives of

the cyanobacteria−sponge symbionts. BLAST similarity searches were also conducted for each cyanobacterial sequence to retrieve the closely related sequences available in the databank. Chimeras and the DGGE clones that did not retrieve sequences similar to *Cyanobacteria* through the BLASTn search in the NCBI database (March 2013) were not included in the phylogenetic analysis. The sequences were aligned with Clustal Omega (Sievers & Higgins, 2014), a multiple sequence alignment program implemented in Sea View v.4.4.2 (Gouy et al., 2010). Ambiguously aligned regions were filtered by Gblocks using less stringent options (Castresana, 2000). A final multiple alignment containing 1293 positions was used for the phylogenetic reconstructions of the 16S rRNA nucleotide data set performed using the Maximum Likelihood (ML) approach (Felsenstein, 1981b) implemented in PhyML (Guindon & Gascuel, 2003a) with a nearest-neighbour-interchange (NNI) heuristic search method, resampled using 100 bootstrap replicates. Posterior probabilities of branch nodes were calculated in MrBayes (BY) v.3.2.6 (Huelsenbeck et al., 2001a) employing the optimal nucleotide substitution model. The best fit evolutionary model– general time reversible (GTR) plus gamma distributed (G) plus invariant sites (I) (GTR+G+I) – was adopted under Akaike's information criterion with correction (AICc) implemented in MrAIC v.1.4.4 (Nylander, 2004).

Results

Isolation of cyanobacteria

To promote growth of the highest diversity possible, 2 isolation media with different compositions were used (MN and Z8 30‰). Eight strains of C*yanobacteria* were isolated from the sponge tissue (Figure 3-2), as well as 1 cyanobacterium from the surrounding waters (*Cyanobium* sp. LEGE10378). These strains belong to the order *Synechococcales* (Table 3-2). In most cases, morphological characterization based on light microscopy allowed identification to genus level, and in some cases to species level. The *Chroococcales* isolates belong to the genera *Synechocystis*. Partial 16S rRNA gene sequences obtained from the isolates were compared with those available in the NCBI database (June 2016), and the results are shown in Table 3-2. Similarities above 98% were obtained for all isolates. The molecular analyses were, in most cases, in agreement with the morphological classification previously done.

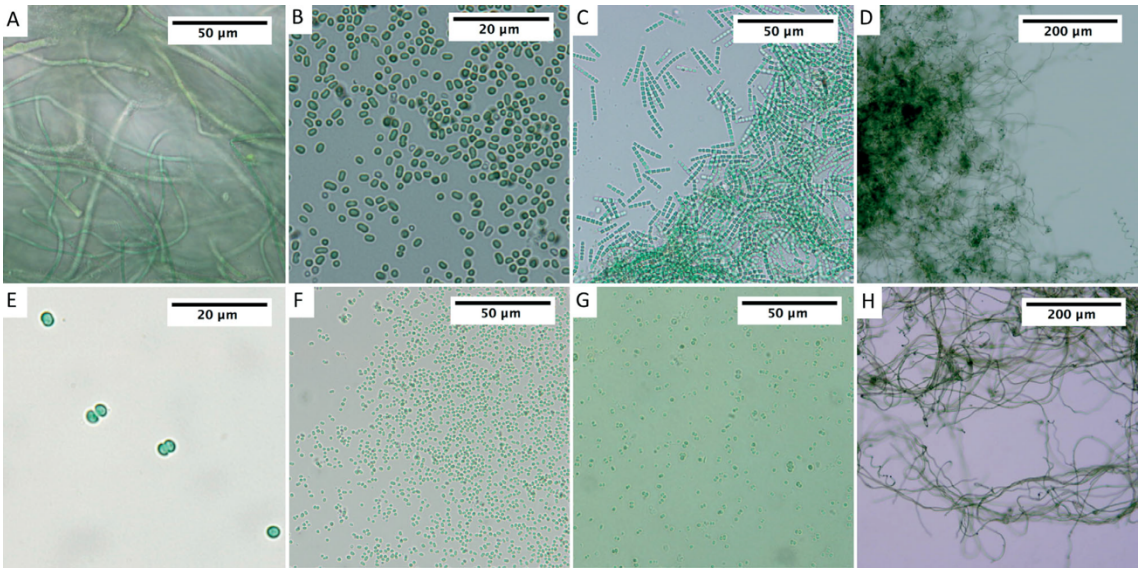

Figure 3-2. *Cyanobacteria* isolated from *Hymeniacidon perlevis.* Identification was done based on morphological characters; accordingly, strains were classified as: (A) *Phormidesmis* sp. LEGE 10370, (B) *Cyanobium* sp. LEGE 11382, (C) *Pseudanabaena* cf. *curta* LEGE 10371, (D) *Nodosilinea* cf. *nodulosa* LEGE 10376, (E) *Synechocystis* sp. 12A21hp, (F) *Synechococcus* sp. 12A10hp, (G) *Cyanobium* sp. 19B10hp and (H) *Nodosilinea* sp. 19D10hp.

Table 3-2. Morphological identification and molecular analysis of the cyanobacterial isolates

Strain reference	*Cyanobacteria* species	Accession no.	Source	Sampling location	Best hit indicated by BLAST		
					Accession no.	Molecular analysis	% max. identity
LEGE 10370	*Phormidesmis* sp.	JQ927344	Sponge	Memória	AY493587	*Pseudophormidium* sp. ANT.LPE.3	98
LEGE 10371	*Pseudanabaena* cf. *curta*	JQ927345	Sponge	Angeiras	AB039018	*Pseudanabaena* sp. PCC7367	98
LEGE 10376	*Nodosilinea* cf. *nodulosa*	JQ927348	Sponge	Porto côvo	EF122600	*Nodosilinea nodulosa* UTEX 2910	99
					HM217060	*Leptolyngbya* sp. LEGE 06308	99
LEGE 10378	*Cyanobium* sp.	JQ927350	Seawater	Aguda	AY172837	*Cyanobium* sp. NS01	99
					HM217069	*Cyanobium* sp. LEGE 06068	99
LEGE 11382	*Cyanobium* sp.	JQ927353	Sponge	Memória	AY172837	*Cyanobium* sp. NS01	100
					HM217069	*Cyanobium* sp. LEGE 06068	100
12A210hp	*Synechocystis* sp.	KX608887	Sponge	Memória	GQ131855	*Synechocystis* sp. *CK5*	98
12A10hp	*Synechococcus* sp.	KX608888	Sponge	Memória	AY172800	*Synechococcus* sp. *ALMO3*	99.8
19B10hp	*Cyanobium* sp.	KX608889	Sponge	Aguda	KC469573	*Cyanobium* sp. *LEGE 06134*	100
19D10hp	*Nodosilinea* sp.	KX608890	Sponge	Aguda	LN849925	*Nodosilinea* sp. *LD14*	98.6

Chl *a* quantification

The method we used for chl *a* quantification in marine sponges has been used by different authors (Becerro & Paul, 2004, Thacker, 2005, Erwin & Thacker, 2007, Thacker et al., 2007, Erwin et al., 2012, Pita et al., 2013, Burgsdorf et al., 2014). Chl *a* quantification was done in sponges collected from different light intensity locations. Chl *a* varied from 6.04 to 17.35 µg g^{-1}, averaging 9,41±3,66(SD) µg g^{-1} of wet sponge. Organic solvents, such as acetone, can interfere with chlorophyll quantification. Chl *d* is

a red-shifted chlorophyll, but when extraction is done with an organic solvent it shows a minor red-shift (Li et al., 2012) and the peaks of chl a and chl *d* overlap. This makes it impossible to distinguish between chl *a* and *d*.

DGGE analysis

A 16S rRNA DGGE analysis was done followed by cloning of the extracted bands and sequencing. In this analysis, both sponge tissues and water samples from the same locations and dates were analysed (Memória, September 2010; Aguda, October 2010; Porto Côvo, November 2010; Memória, September 2011). To analyse the banding pattern, we assumed that bands in the same position on the gel represented the same organism. We were able to determine the presence of 24 unique bands from DGGE (Figure 3-3). Ten of them were present in all samples, both from sponge tissues and water samples (stars (⋆) in Figure 3-3). Another 10 were found exclusively in water samples (triangles (▶) in Figure 3-3) and 4 in sponge tissue only (filled circles (●) in Figure 3-3). Because a single DGGE band can represent more than one strain, we cloned each extracted DGGE band twice. From the initial 24 bands, we successfully identified 10 clones corresponding to 9 different bands (numbered 1 to 9 in Figure 3-3). The closest representatives of the clones retrieved through the BLASTn search are shown in Table 3-3. DGGE banding patterns revealed higher species richness in the seawater compared to the sponge samples (Figure 3-3). DGGE clone 1_1, derived from a band only present in sponge tissue, showed molecular similarity with other *Cyanobacteria* identified in the sponge *Hymeniacidon heliophila*. The same similarity was found in DGGE clone 7_1, extracted from a band only present in water samples. Two of them (DGGE clones 4_1 and 5_1) seem to belong to the genus *Acaryochloris*, and clones 2_1, 6_1, 8_2 and 9_1 to the genus *Synechococcus*.

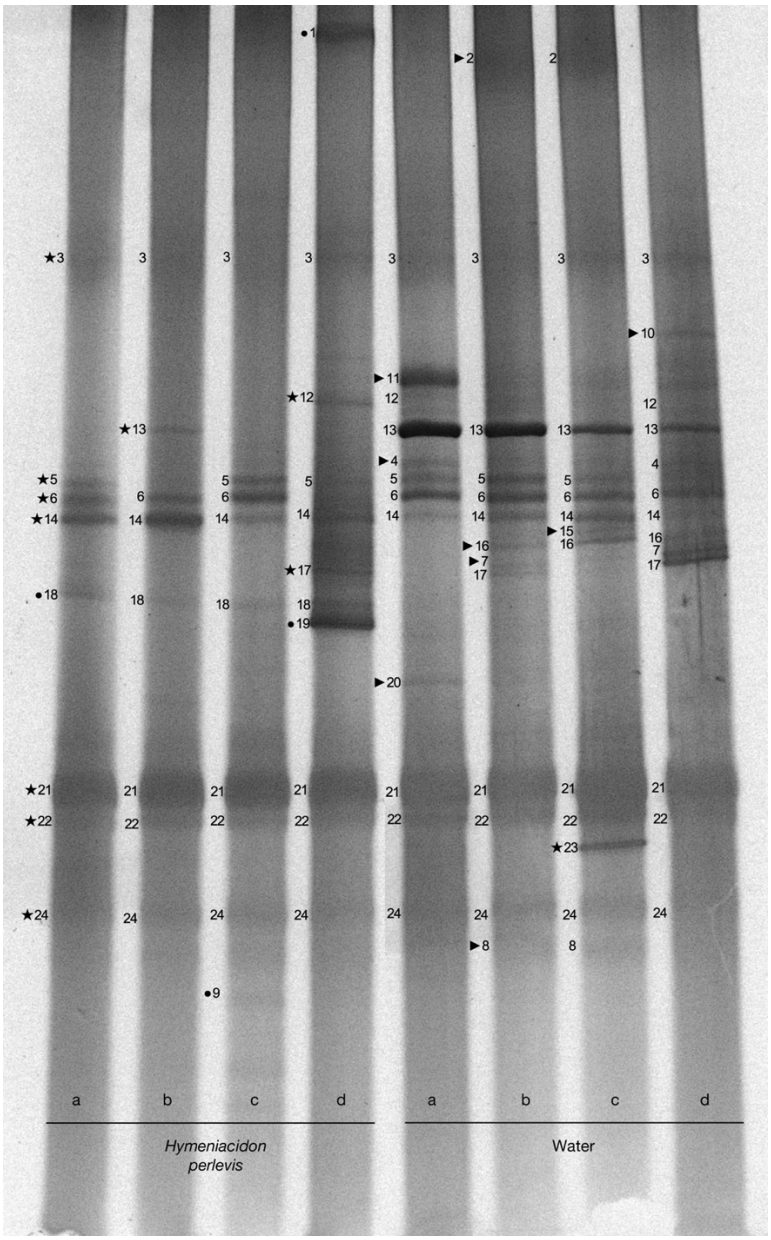

Figure 3-3. Denaturing gradient gel electrophoresis (DGGE) banding profiles of cyanobacterial 16S rRNA genes PCR-amplified from the tissue of the marine sponge *Hymeniacidon perlevis* tissue in comparison to samples of seawater from same locations and dates (a−d). (a) Memória (September 2010); (b) Aguda (October 2010); (c) Porto Côvo (November 2010); (d) Memória (September 2011). Individual bands are labelled on the left-hand side of the lane numbered from 1 to 24. (►) bands present only in water samples; (●) bands present only in sponge samples; (★) bands present both in water and sponge samples.

Phylogenetic analysis

Phylogenetic analysis was performed to assess the relative positioning of the isolated cyanobacteria and DGGE clones from the present study with free-living and previously reported sponge-associated cyanobacteria. The phylogenetic tree (Figure 3-4) revealed a well-supported topology, both by ML and Bayesian tree-reconstruction approaches, showing a heterogeneous diversity among our sequences, clearly forming 3 distinct

clusters. DGGE clones and most of the cyanobacterial isolates from the sponges grouped in cluster A, which was mainly comprised of unicellular cyanobacteria and the filamentous *Pseudanabaena* genus. The isolate from seawater (*Cyanobium* sp. LEGE 10378) was also placed in this cluster. DGGE clones in cluster A showed similarity both with previously reported sponge-associated cyanobacteria and free-living strains. Two DGGE clones (clone 4_1 and clone 5_1) showed similarity with *Acaryochloris* sp., one (clone 8_2) with *Prochlorococcus* sp., 5 (clones 1_1, 2_1, 6_1, 7_1 and 9_1) with *Synechococcus* sp., and the remaining 2 (clones 3_1 and 8_1) had affiliation with *Synechocystis* sp.. Clusters B and C were mainly comprised of filamentous species. Cluster B grouped *Phormidesmis* sp. (LEGE10370) with different filamentous cyanobacteria, as well as a *Synechococcus* species. Cluster C only contained *Nodosilinea* species.

Table 3-3. Phylogenetic affiliations of 16S rRNA gene clones obtained from denaturing gradient gel electrophoresis (DGGE) bands from *Hymeniacidon perlevis* and seawater

DGGE clone	Accession no.	Source	Best hit indicated by BLAST		
			Accession no.	Molecular analysis	% max. identity
1_1	KC896629	Sponge	JF824768	Uncultured cyanobacterium from *Hymeniacidon heliophila*	99
2_1	KC896630	Seawater	AY172835	*Synechococcus* sp. WH8020	99
3_1	KC896631	Seawater	JN825316	Uncultured cyanobacterium	99
4_1	KC896632	Seawater	NR_074407	*Acaryochloris marina* MBIC11017	99
5_1	KC896633	Sponge	NR_074407	*Acaryochloris marina* MBIC11017	99
6_1	KC896634	Sponge	HE687328	Uncultured *Synechococcus*	99
7_1	KC896635	Seawater	AM259807	Uncultured cyanobacterium from *Thethya aurantium*	99
8_1	KC896636	Seawater	JX255822	Uncultured cyanobacterium	99
8_2	KC896637	Seawater	FJ903249	Uncultured *Synechococcus*	99
9_1	KC896638	Sponge	HE687328	Uncultured *Synechococcus*	99

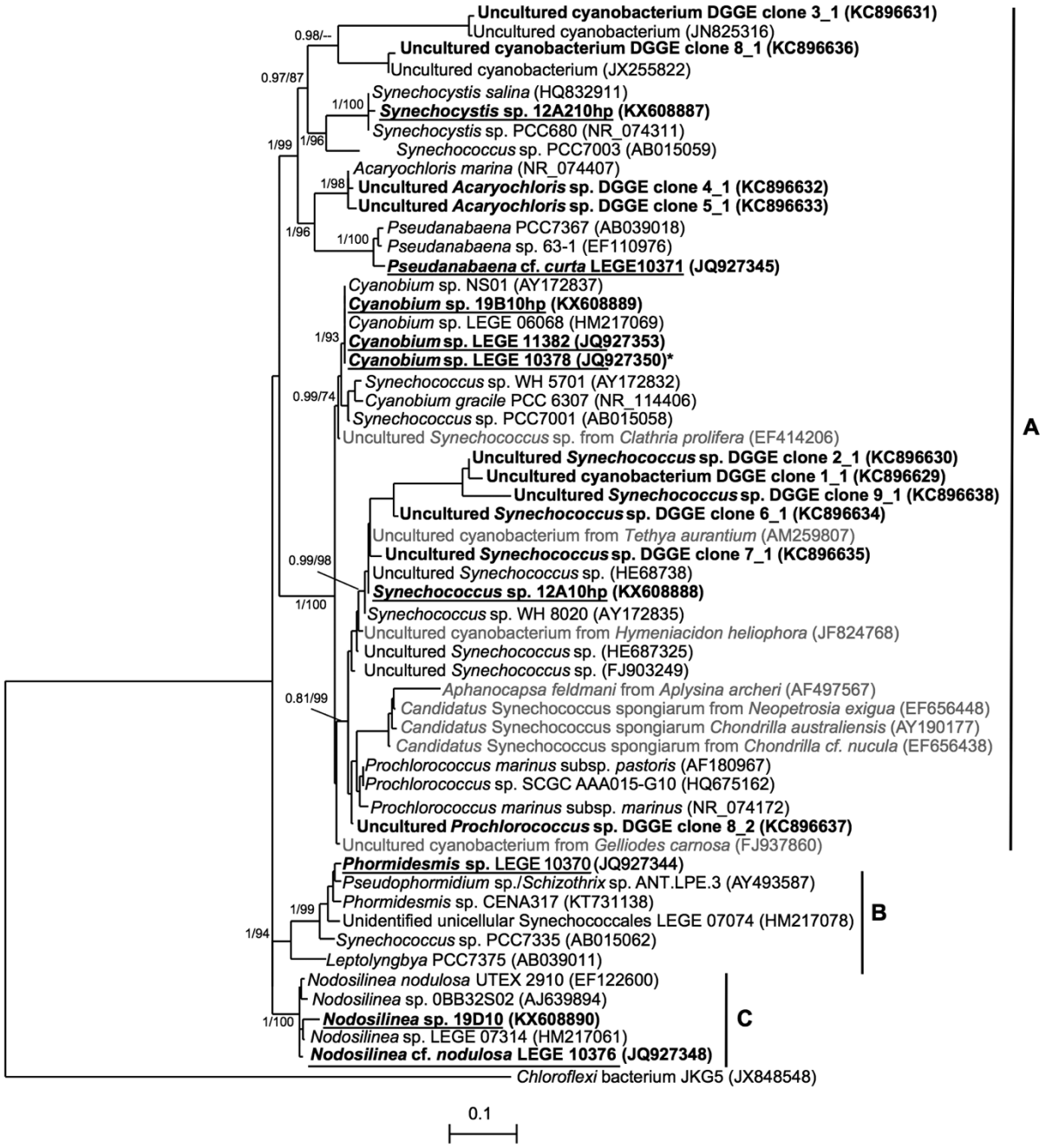

Figure 3-4. Maximum likelihood (ML) phylogenetic tree based on the 16S rRNA sequences. The isolates from the present study are in bold and underlined. Isolate with an asterisk (*) was isolated from water sample. Denaturing gradient gel electrophoresis (DGGE) clones obtained from the present study are in bold. The different cyanobacterial clusters are represented with letters from A to C. 16S rRNA sequences obtained from marine sponges are in grey with information of the host sponge species. The retrieved sequences of GenBank were selected based on being the reference strains and the best match for BLASTn analysis. GenBank accession numbers are given in parentheses. The tree was rooted using *Chloroflexi* bacterium JKG5. Bayesian posterior probabilities and ML bootstrap support values are represented at the nodes. Only bootstrap values greater than 50% are given. The scale bar at the bottom represents 10% sequence divergence

Discussion

Hymeniacidon perlevis is one of the most common sponge species along the rocky intertidal shore of Portugal. The presence of photosymbionts such as *Cyanobacteria* may be beneficial for the survival and growth of this sponge. In this study, we assessed the cyanobacterial diversity contained in *H. perlevis* sampled from the coast of Portugal (Figure 3-1), using culture-dependent and culture-independent approaches. Although photosynthetic microorganisms are usually present in the outer layers (which are more exposed to sunlight) while the inner layers (mesohyl) are populated by heterotrophic and autotrophic bacteria (Hentschel et al., 2003, Kennedy et al., 2007), *Cyanobacteria* are distributed throughout the whole sponge, as the mesohyl provides higher quantities of nutrients than the surrounding waters (Hentschel et al., 2006, Kennedy et al., 2007). Hence, the whole sponge tissue was used to isolate and assess cyanobacterial diversity. We succeeded in characterizing 8 isolates, 7 of them from sponge tissue using phenotypic characteristics (Figure 3-2) and molecular features. Phylogenetic analysis of the cyanobacterial isolates from this study (Figure 3-4) were in agreement with the morphological characters validating their taxonomic affiliation (Komárek et al., 2014).

Erwin and Thacker (2007) classified sponges according to their photosynthetic community through chl *a* quantification. The sponges from the present study might harbour a small photosynthetic community, as the values determined for all 9 specimens were below 50 μg g^{-1}. As previously noted, the results for chl *a* quantification may also be incorporating chl *d* (Li et al., 2012). Through DGGE analysis, we were not able to identify *Synechococcus spongiarum*, known to be a true sponge cyanobiont. Erwin and Thacker (2007) reported that low chl *a* sponges did not harbour *S. spongiarum*. DGGE analysis only showed the presence of unicellular cyanobacteria, indicating that these are likely more abundant than filamentous forms.

Molecular analysis revealed different DGGE banding patterns between seawater and sponge samples (Figure 3-3), suggesting the presence of sponge-associated cyanobacterial communities that are distinct from the seawater. Interestingly, the 16S rRNA DGGE fingerprint from *H. perlevis* samples a, b and c (Figure 3-3), sampled from different geographical locations within an interval of 3 months, revealed a similar banding pattern, further pointing to a consistency in sponge-associated cyanobacteria, even though there was a slight change in the banding pattern from the seawater. However, we observed an enrichment of the cyanobacterial community, represented by the

presence of more bands, in sponge-d compared to sponge-a, which were collected from the same location at times 1 year apart. This observed trend of inconsistency among the sponge-associated bacteria has been previously reported, suggesting the possibility of temporary association or host-switching (Alex et al., 2012), or part of the sponges' dietary supply (Sipkema et al., 2015). It is known that irradiance conditions may influence the photosynthetic activity of sponge-associated cyanobacteria (Erwin et al., 2012), which could explain the differences in the banding patterns between sponges a and d.

Not all filtered bacteria are ingested. They can survive and grow in the mesohyl tissue, becoming part of the sponge microbial community (Kennedy et al., 2007). It is known that only the most common and abundant organisms of the main populations are displayed in the banding pattern, and that organisms representing <1% of the community will not be identified (Muyzer et al., 1993), resulting in an underestimation of the bacterial community. Many bands that were present in sponge samples were not detected in the water samples, and recent studies have shown that most of the sponge-specific 16S rRNA gene sequence clusters are also present in the seawater but in smaller amounts (Taylor et al., 2013). The absence of a band in the DGGE does not necessarily mean the absence of that species; rather, it could mean that the organism was present at the moment of collection, but in an amount below the detection limit of the method. Banding pattern shifts cannot be analysed in terms of diversity, only abundance. Some bands, more evident in the sponge tissues than water samples, may show a selective uptake of the cyanobacterium.

From the 24 bands present in DGGE, only 10 were successfully sequenced. This can explain why filamentous cyanobacteria were not identified. Also, filamentous cyanobacteria may exist in smaller amounts than the detection limit of the method. In the future, it would be interesting to clone more bands, as well as to pick more clones from each band, to assess cyanobacterial diversity.

The phylogenetic analysis with partial 16S rRNA gene sequences from the isolates and DGGE band clones show 3 different clusters. Clusters A and C were comprised of only *Synechococcales*, and cluster B of *Synechococcales* and an *Oscillatoriales* (Figure 3-4). Sponge-associated cyanobacteria from our study resulted in polyphyletic clusters, which is a common phenomenon according to previous reports (e.g. Steindler et al. (2005)). Although we failed to detect *S. spongiarum*, in accordance with a previous study our phylogenetic reconstruction showed a clear distinction between free-living cyanobacteria and the cyanobionts (*S. spongiarum*) (cluster A; Figure 3-4) (Erwin & Thacker, 2007). Cluster A was represented by *Synechococcus* and *Prochlorococcus* species, an

association that has been widely described in 26 Demospongiae and 17 Calcarea families (Li et al., 2011). An earlier study also showed that the marine sponge *Clathria prolifera* harboured cyanobacteria belonging to the genus *Pseudanabaena* (Isaacs et al., 2009). All DGGE clones are represented in cluster A.

Retrieval of DGGE clones from sponge tissue with significant similarity to *Acaryochloris marina* further validated *Acaryochloris* as a *H. perlevis*-associated cyanobacterium, which has been reported previously in sponges (Alex et al., 2012) and sea-squirts (López-Legentil et al., 2011). *Acaryochloris* is the only known producer of chl *d*, a red-shift chlorophyll. Chl *d* was first identified in red algae (Manning & Strain, 1943), then in *A. marina* (Miyashita et al., 1996); eventually, *Acaryochloris* was confirmed as the only chl *d* producer (Murakami et al., 2004). Chl *d* in this cyanobacterium accounts for 95 to 99% of all chlorophyll content (Miyashita et al., 1996), replacing all function of chl *a* and allowing it to exploit light environments depleted of visible radiation. Due to its unique use of far-red light, *Acaryochloris* can live in niches in coastal waters (Murakami et al., 2004), and therefore its presence in intertidal marine sponges is expected, due to their filtration capability. The association of sponges with *Acaryochloris* can be beneficial due to this red-shift chlorophyll. In order to confirm the presence of *Acaryochloris* in *H. perlevis*, in the future, it would be interesting to quantify both chl *a* and chl *d* using the methods described by Li et al. (2012).

Cluster B comprised species from the genera *Leptolyngbya*, *Phormidesmis* and *Pseudophormidium*, as well as a strain from *Synechococcus* and a former *Synechococcus*, now identified as unicellular Synechococcales. The clustering of *Synechococcus* sp. PCC 7335 with filamentous non-heterocystous cyanobacteria from the genus *Leptolyngbya* has been previously reported (Honda et al., 1999, Castenholz et al., 2001, Wilmotte & Herdman, 2001). Cluster C formed a well-supported group, only containing *Nodosilinea* species.

The presence of sponge-associated cyanobacteria from seawater samples supports the hypothesis of procurement of symbionts through the environment, i.e. horizontal transmission (Maldonado, 2007), apart from the commonly accepted vertical mode of transmission. Furthermore, it indicates the ability of sponge-associated cyanobacteria to survive outside the host tissue (Taylor et al., 2013). According to Alex et al. (2013), *H. perlevis* from the Portuguese coast is a LMA sponge, and it has been suggested by Giles et al. (2013) that LMA sponges may acquire bacteria mainly from ambient seawater. In addition, sponges are filter-feeding animals that use picoplanktonic cyanobacteria as a source of food. Due to the close phylogenetic relationship to planktonic *Synechococcus*

strains, a seawater origin for the *H. perlevis* cyanobacterial clones cannot be excluded. But it can also point to the existence of a community shared between sponges and the surrounding waters, because it is known that the sponge microbial community is a mixture of organisms acquired both from the water column and by vertical transmission (Hentschel et al., 2003). Usher et al. (2001) observed *Cyanobacteria* in only 25% of sponge larvae, suggesting that vertical transmission is not the only mode of symbiont procurement. Bacterial profile assessment and comparison using adults and embryos could further validate the mode of symbiont transmission among the intertidal sponge *H. perlevis*.

As has been previously reported (Steindler et al., 2002), the presence of *Cyanobacteria* can be very important for the survival of intertidal marine sponges. For instance, these sponges are prone to air exposure, leading to fluctuations in temperature and irradiance, and lack of filter feeding opportunities (Steindler et al., 2002). During these conditions, the photosymbionts play an important role, providing the sponge hosts with nutrient uptake for their survival and the production of potential UV-screening substances (Steindler et al., 2002). Although we employed a relatively inexpensive technique (DGGE) to profile the microbial diversity, it provided a first glimpse of the cyanobacterial community, allowing visualization and monitoring of changes directly from the banding patterns. This method, when used for a long period can also allow differentiation between transient and permanent communities (Hentschel et al., 2003). Further determination of the origin and diversity of sponge-associated cyanobacteria in comparison to their free-living counterparts can be achieved using advanced next-generation sequencing techniques.

Many previous studies have reported the presence of a huge diversity of marine cyanobacteria isolated from the Portuguese coast (e.g. Brito et al. (2012), (Leão et al., 2013)). Brito et al. (2012) reported that *Cyanobium*, *Leptolyngbya* and *Pseudanabaena* were the most abundant genera among isolates. Strains from the same genera obtained in the present study, also collected from the coast of Portugal, were found to be a source of bioactive compounds (Leão et al., 2013, Costa et al., 2014, Brito et al., 2015, Costa et al., 2015) namely strains from the genera *Cyanobium* (Costa et al., 2015), *Leptolyngbya*, *Synechocystis*, *Nodosilinea* and *Pseudanabaena* (Costa et al., 2014). Isolation and growth of these species under laboratory conditions would be necessary to obtain sufficient quantities of these natural compounds for their detailed chemical characterisation and production. Also, due to the negligible amount of some cyanobacteria in seawater, they could easily be missed when isolating and culturing, and

hence their bioactive potential would remain unexploited. Sponges are filter-feeders capable of pumping 24cm^3 of seawater per day, per kg of sponge (Vogel, 1977), with very efficient filtration systems and a clearance rate of up to 61% (Stabili et al., 2006). In this way, sponges could be used as a natural filtration and concentration mechanism to obtain new cyanobacterial strains with pharmaceutical potential.

The present study shows, for the first time, the diversity of cyanobacteria associated with marine sponges from the intertidal area of the Portuguese coast (NE Atlantic) using both culture- and molecular-based methods, and the comparison of the sponges' cyanobacterial community to that present in seawater. Even although the true cyanobacterial diversity might be underestimated, culture-dependent and culture-independent methods showed that some sponge-associated cyanobacteria were detected in the surrounding waters, suggesting temporary or selective uptake. Nevertheless, we argue that the recurrent presence of a cyanobacterial community at different spatial and temporal scales could be indicative of environmental acquisition of *Cyanobacteria* by the intertidal marine sponge *H. perlevis*. Finally, the isolation technique employed here could be used to isolate new cyanobacteria that are only present in small amounts in the water column.

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Chapter 4. Changes in the bacterial community of the marine sponge *Hymeniacidon perlevis* from *in situ* and *ex situ* conditions: insights on the cyanobacterial diversity

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Abstract

The microbial community in marine sponges have been extendedly studied in the last few decades, with NGS approaches providing new insights to these associations. Several experiments have the need to maintain sponges in *ex situ* conditions, which is known to affect their microbial community and even sponge survival. In the present work, a 454-pyrosequencing analysis was conducted on the sponge *H. perlevis*, both from *in situ* and after maintenance *ex situ*. Results showed the diference in bacterial community between the sponges and natural seawater. In sponges, Proteobacteria was a major phylum present in all sponge samples. Some organisms, such as Cyanobacteria almost disappeared from sponge tissue under controlled conditions, after 30 days. TEM analysis from sponge tissue also showed the same cyanobacterial trend. We hypothesized that sponge viability was compromised by the loss of cyanobionts. In terms of community diversity and richness, all sponge samples showed similarities, but when applied a beta-diversity analysis it was evident how the community changed along the time frame under *ex situ* conditions. This work shows the need to study the bacterial community and its balance prior to conduct more extensive studies and further investigations must be made in order to confirm the results here presented.

Keywords

Hymeniacidon perlevis; Porifera; pyrosequencing; bacterial diversity; *ex situ* maintenance; cyanobacteria

Introduction

Sponges, Phylum Porifera, have fossil records dating back to around 580 million years (Hentschel et al., 2006), and constitute the bottom (less evolved) of the Metazoan branch. Although sessile animals, they are present in every aquatic environment, at all depths (Sarà & Vacelet, 1973, Bergquist, 1978, Van Soest et al., 2012), with a huge

diversity in number of species and morphological characters. Characterized by a simple body plan, highly totipotent cells, a characteristic aquiferous system and different reproduction strategies, their lifestyle has proven to be very successful. In marine environments, sponges play important roles in the cycle processes of dissolved nutrients and organic matter (Maldonado & Riesgo, 2008), and are a vast source of compounds with biotechnological applications (Leal et al., 2012).

Mainly in the mesohyl tissue, inhabit a huge diversity of microorganisms comprising as much as 40% of the total sponge volume (Vacelet, 1975, Vacelet & Donadey, 1977, Webster & Taylor, 2012). Many of these associations, evolved millions of years ago playing an important role in both sponge survival and evolution (Taylor et al., 2007b). The sponge benefits from these associations, through translocation of metabolites from microorganisms in the form of glycerol (Wilkinson & Fay, 1979), organic phosphate and nitrogen (Wilkinson & Fay, 1979) or glucose (Wilkinson, 1980), which is known to enhance sponge growth rate and competitiveness with other benthic communities (Wilkinson, 1980, Arillo et al., 1993). Bacteria can also participate in chemical defence of the host against both predators and biofouling (Unson et al., 1994, Schmidt et al., 2000). It has also been proven that sponge survival, in many cases, can be directly linked to the stability of certain symbionts. For example, Thacker (2005), observed that a decline on the cyanobacterial community of the sponge was related with a decrease of sponge health. Translocation of sponges to *ex situ* conditions can have implications in their microbial community, and consequently in the survival of the sponge.

Addressing microbial diversity using culture independent techniques was a major breakthrough, leading to the discovery of many more phyla. Hentschel et al. (2002) compared the microbial community between sponges, surrounding water and sediment, showing for the first time the evidence of a monophyletic, sponge specific clusters and a uniform bacterial community in marine sponges on a global scale. As early as in the 70's, Wilkinson (1978b) stated that the microbial community presented in sponges were very different from the one presented in surrounded seawater. Molecular based techniques were able to confirm that statement (Hentschel et al., 2006, Taylor et al., 2007a, Hardoim et al., 2009, Hentschel et al., 2012, Webster & Taylor, 2012).

The use of high-throughput sequencing techniques such as next generation sequencing (NGS) 454-pyrosequencing provided, in the last decade, new insights in sponge microbiology.

Through NGS studies, different new phyla were unveiled (Cárdenas et al., 2014, Hardoim et al., 2014, Kennedy et al., 2014, Naim et al., 2014), concluding that bacterial communities were species specific (Lee et al., 2011) and the presence of a low core

community (<1%) (Schmitt et al., 2012), against the previous idea of a sponge specific community across different sponges. As part of the global sponge microbiome project, it has been found more than 40 microbial phyla or candidate phyla in sponges, where most OTUs (operational taxonomic units) were present in a small fraction of the sponges, and only a few found in most sponge species (Thomas et al., 2016, Moitinho-Silva et al., 2017). Apart from revealing new phyla, these works showed that the dominant bacterial taxa were the same as the ones described in previous studies using 16S rRNA gene libraries: Proteobacteria (Gamma- and Alpha-), Actinobacteria, Chloroflexi, Nitrospirae, Cyanobacteria and Candidatus Phylum Poribacteria (Pita et al., 2018).

The study of the sponge-microbe symbiotic community can help to understand the diversity of Proto-Eukaryote symbiosis. Also, despite their simple body plan, sponges are situated in an important phylogenetic position (bottom of the Metazoan) among marine invertebrates, making them ideal candidates as animal models. Porifera has a huge genomic complexity (Riesgo et al., 2014), expressing homologs of genes involved in the animal nervous system (Ludeman et al., 2014) and in innate immunity (Hentschel et al., 2012, Riesgo et al., 2014).

Many studies are now focusing also in the ability of sponges and their symbionts to produce secondary metabolites (toxins and compounds with pharmaceutical interest). Translocation of sponges from natural environment to laboratory-controlled conditions can be necessary for several studies, which may influence the symbiotic community. The aim of this study is to assess the microbial community in the marine sponge *Hymeniacidon perlevis*, a common intertidal marine sponge of the Portuguese coast, and to understand how the bacterial community is affected by translocation to *ex situ* conditions. With that intent, we aim to perform a 454-pyrosequencing analysis from sponge tissue collected *in situ* and *ex situ.* Those results will be combined with information from TEM (transmission electron microscopy) analysis, in order to infer on changes of the bacterial community, and especially within the cyanobacterial community

Materials and Methods

Sample collection, preparation and aquarium maintenance

A specimen of the marine sponge *Hymeniacidon perlevis* (Montagu, 1814) (Figure 4-1) were collected from the intertidal area of Memória beach, Matosinhos Portugal (Figure 4-2). Memória beach has a combination of sand and granite rocks and the coast is exposed to the prevailing northwest oceanic swell, which can reach values over 5m in the winter, and sea surface temperature ranging from 13-20 ºC. The tidal regime along

the Portuguese coast is semi-diurnal with the largest tidal range during spring tides of 3.5 and 4 m.

Sponges were attached to rocky surfaces in sheltered areas, protected from direct influence of the sun and tide, and collected with the help of a knife, cleaned of debris and sediment and placed in sterile 100 mL flasks containing natural seawater from the sampling location. Water sample (2,5 L) was also collected. After collection, sponge samples were immediately transported to the laboratory and processing began as soon as arriving. A small fraction of *H. perlevis* (1 cm^3) was preserved in 100% ethanol for subsequent molecular analysis and morphological identification; approximately 2 mm of the sponge was cut for transmission electron microscopy use. Seawater was filtered through a 0.45 µm sterile filter followed by DNA extraction.

Sponge identification was based on the sampling habitat, shape, consistency, texture, colour, smell and characteristic features (morphology, dimensions) of spicules (Hooper & van Soest, 2002).

The collected specimen was placed in containers with natural filtered seawater (2 µm net pore) for a period of 2 hours. After each 20 minutes the water was changed. This process helped in removing transient microorganisms or debris from the sponge. Sponge were transferred to 30 L aquariums with 15 L of natural seawater obtained from a place near where the specimens were collected. Prior to use, natural seawater was submitted to a 24 h sedimentation process, followed by two filtration steps (100 µm, and 25 µm). The aquarium was equipped with a filtration system (Boyu Model FP- 28E). Aquarium room was kept at a temperature of 16 ºC and salinity, temperature, dissolved oxygen and pH were quantified every day. Aquarium water was changed every week. Sponges were fed every day with a 5 mL solution composed by two commercial aquarium foods: 1 full spoon (provided with the commercial food) of Tropic Marin® Pro-Coral Zooton and half a spoon of Cyclop-Eeze (Argent Chemical Laboratories). Prior to feeding process the filtration system was turned off and kept off for 10 minutes. The solution was provided with the help of a sterile pipette and deposited on the sponge surface. Sponge was kept under aquarium conditions for a total period of 30 days. After 15 and 30 days fragments of the sponge were collected, both for molecular analysis and for TEM.

After 30 days, *H. perlevis* started losing their characteristics. Lost color and structure, leading to lose of viability, stopping the experiment.

Figure 4-1.Picture of a specimen of *Hymeniacidon perlevis* in their natural habitat

Figure 4-2. Sampling location: Memória (41° 13' 52.27''N, 8° 43' 18.34''W), with pictures of the sampling area.

Transmission Electron Microscopy

Sponge tissue (~2 mm) was cut and immediately fixed in 2% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 2 h. After that it was washed three times in double strength buffer, post-fixed with 2% osmium tetroxide in 50 mM sodium cacodylate buffer (pH 7.2) for 2 h, and washed again in double strength buffer. The dehydration was performed using an ethanol series (25–100%; v/v), and once using propylene oxide. Samples were embedded in mixtures of propylene oxide and Epon resin, followed by Epon for at least 24 h, before being placed in embedding moulds with Epon, and being allowed to polymerize at 55 °C. Thin sections were cut with a Leica Reichert Supernova ultramicrotome, and mounted in copper grids 200 Mesh. The sections were contrasted before being visualized using a transmission electron microscope Jeol JEM 1400 operating at 80 kV (IBMC / HEMS).

Molecular analyses and 454 pyrosequencing

Total genomic DNA (gDNA) was extracted from sponge tissue and sampled seawater, using a commercially available Purelink^{TM} genomic DNA kit (Invitrogen) and stored at -20°C until further analysis.

The 16S rDNA of each sample was amplified and barcoded for pyrosequencing using a 10 bp barcode sequence (Table 4-1) added to the polymerase chain reaction (PCR) primers: forward primer U789F (5′-TAGATACCCSSGTAGTCC-3′) and reverse primer U1068R (5′-CTGACGRCRGCCATGC-3′) (Baker et al., 2003). PCR reactions were performed in triplicates (final volume of 100 µL) containing 5 U of Pfx50 DNA polymerase (Invitrogen), 1X Pfx50 PCR mix, 0.3 mM of dNTPs (NZYTech), 0.5 µM of each barcoded primer and 30 ng of metagenomic DNA. PCR reaction started with an initial denaturation at 95 ºC for 5 minutes, followed by 26 cycles of 94 °C for 15 seconds, 63 °C for 30 seconds, 68 °C for 45 seconds, and a final extension step at 68 ºC for 5 minutes. PCR products were purified using a PCR gel extraction purification kit (Macherey-Nagel). Products were pooled on a titanium adaptor and pyrosequencing was performed on ROCHE 454 GS-FLX Titanium platform. Raw pyrosequencing reads were submitted to the NCBI Short Reads Archive database (SRR949132).

Table 4-1. List of samples with the respective sample codes and multiplex identifiers (MID)

List of samples	Sample code	MID
Seawater	SW	TAGATACCCSSGTAGTCC
H. perlevis (environmental sample)	Hp	TAGATACCCSSGTAGTCC
H. perlevis (15 days *ex situ*)	Hp15d	TAGATACCCSSGTAGTCC
H. perlevis (30 days *ex situ*)	Hp30d	TAGATACCCSSGTAGTCC

454 tag sequence processing and OTU picking

Pyrosequencing data analysis were performed with The Quantitative Insights Into Microbial Ecology software package (QIIME) v.1.9.1 (Caporaso et al., 2010b). Summarizing, raw multiplexed sequences (34654 reads) were assigned to samples based on barcodes for downstream analysis and pre-processed by trimming with an average quality threshold score of 25, removing reads containing ambiguous bases or where bad windows were found, as well as sequences shorter than 100 bp and unassigned reads. Final sequences were of an average read length of 286.5 bp. After, pre-processed dataset was screened by denoising (Reeder & Knight, 2010) to avoid over representation of species diversity. Operational taxonomical units (OTUs) were determined at 97% sequence similarity using the UCLUST method (Edgar, 2010). Taxonomy assignment of representative sequences of each OTU were picked using

QIIME default parameters, and aligned employing PyNAST (Caporaso et al., 2010a) against a Greengenes core reference alignment (DeSantis et al., 2006). From the OTU table originated, undesirable OTUs were removed (Archaea, and singletons (one single sequence))

A final OTU biom-format table was then created and used as input data for downstream analyses.

Microbial diversity and co-occurrence analysis

After taxonomic assignment using QIIME, bar charts were created at phylum level for each sample. In order to avoid biases related to sequencing depth, libraries were normalized by size through randomly picking sequences that were further used for both alpha- and beta-diversity metrics.

Microbial richness indices namely observed species richness (S.obs), expected richness with Chao1 estimator (S.Chao1) (Chao, 1987), and diversity measure of Shannon indices (Shannon, 1948) were executed in QIIME environment. Measures of (dis)similarity in microbial community composition between samples was made through multivariate analysis of the community composition at the OTU level (97% sequence cut-off) performed using beta-diversity unweighted Unique Fraction metric (UniFrac) (Lozupone & Knight, 2005), and used for multivariate analysis by Principal Coordinate Analysis (PCoA) (Krzanowski & Krzanowski, 2000). To estimate uncertainty in hierarchical clustering and PCoA plots of bacterial communities, a Jackknife beta-diversity analysis was used.

All QIIME scripts used are described as supplementary work.

Results

The present work intended to analyse and compare the bacterial community of a common intertidal marine sponge, *Hymeniacidon perlevis*, and see how maintenance under controlled conditions, *ex situ*, would change the community. Short after 30 days *ex situ*, the viability of the sponge was compromised and died.

From 454 data, we retrieved a total of 12114 16S rRNA V4-tag sequences. After quality sequencing filtering, we obtained 7685 sequences. Table 4-2 summarizes the sequence data, where filtered sequences were assigned to a total of 507 operational taxonomic units (OTUs), at a 97% similarity cut-off.

Table 4-2. Summary of sequence data

Sample	**Sample code**	**Sequences**	**OTUs 97**
Seawater	SW	1699	324
H. perlevis (environmental sample)	Hp	334	58
H. perlevis (15 days *ex situ*)	Hp15d	1914	144
H. perlevis (30 days *ex situ*)	Hp30d	3738	213

Observing the community composition at phylum level, represented in Figure 4-3, it is possible to notice that seawater bacterial composition differs from the ones from sponges. And that translocation to controlled conditions affect the bacterial community in sponges. Phyla with less than 2% of representativeness were grouped together and displayed in Figure 4-3 as “Other”. Those are Actinobacteria, BHI80-139, BRC1, Chlamydiae, Firmicutes, Gemmatimonadetes, Lentisphaerae, Nitrospirae, OP1, OP3, OP8, PAUC34f, SAR406, Synergistetes, TM6, WPS-2, WS2, ZB3 and [Thermi]. Altogether, 31 different phyla were identified, among recognized and candidate taxa. In sponge samples, 21 different phyla were identified. Seawater is dominated by Planctomycetes (155 OTUs and 815 sequences), while sponges by Proteobacteria. Inside the Phylum Proteobacteria, classes Alpha- Delta- and Gamma-Proteobacteria are the most common. The most dominant sponge-associate phyla were Proteobacteria, Planctomycetes, Cyanobacteria and Bacteroidetes. Under controlled conditions, the candidate Phylum SBR1039 started growing and after 30 days *ex situ*, the most important remark was the almost absence of the phylum Cyanobacteria (1 OTU, 3 sequences).

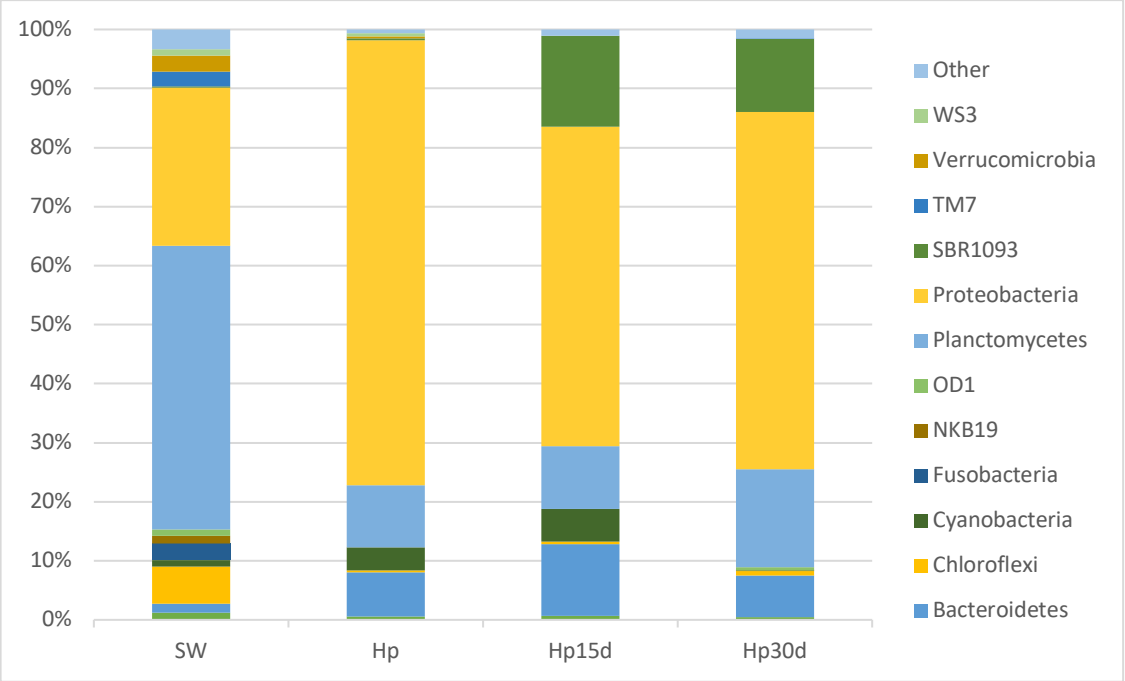

Figure 4-3. Bacterial community at Phylum level for all samples. All Phylum with less than 2% diversity were combined and are represented as other. SW: seawater sample; Hp: *H. perlevis in situ*; Hp15d: *H. perlevis* 15 days *ex situ*, under controlled conditions; Hp30d: *H. perlevis* 30 days *ex situ*, under controlled conditions.

The same pattern was observed through TEM analysis (Figure 4-4). In both the sponge *in situ* (Figure 4-4a-b) and in the sponge after 15 days under controlled conditions (Figure 4-4c-d), unicellular cyanobacteria were observed inside cyanocytes, with the presence of spiral thylakoids. No cyanobacteria were observed in TEM analysis in the sponge after 30 days under controlled conditions.

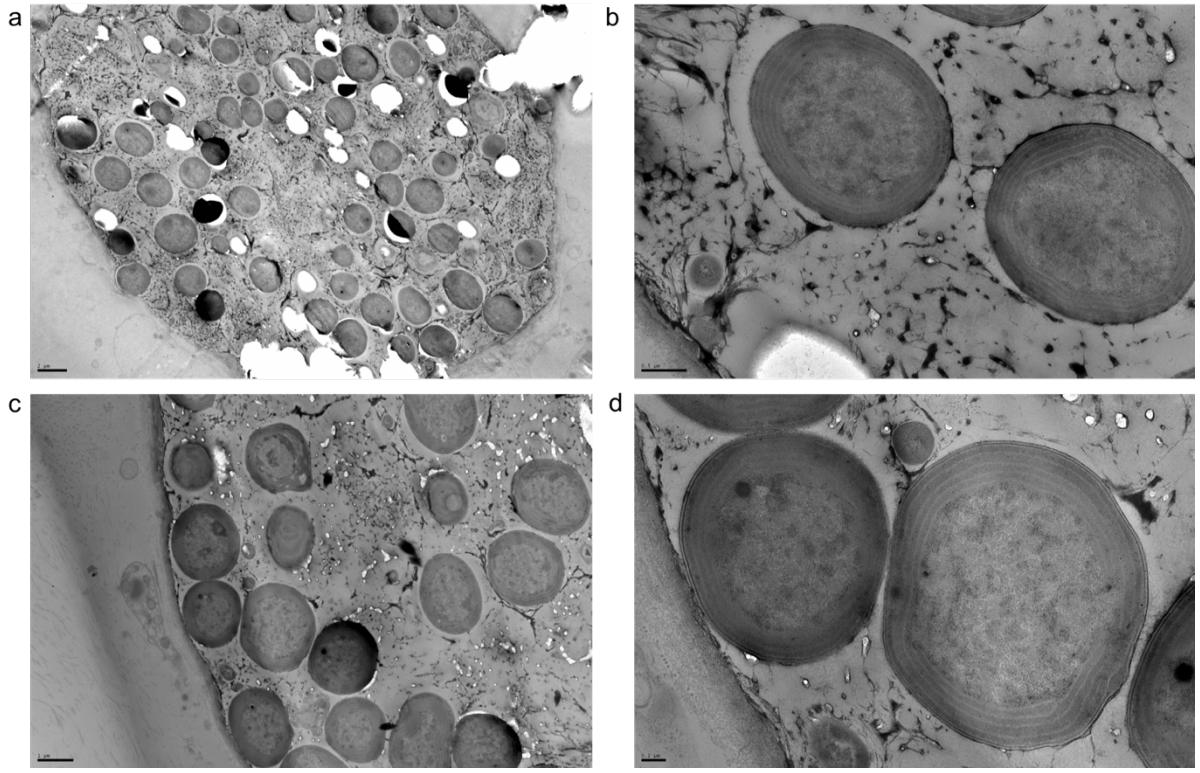

Figure 4-4. Transmission electron microscopy of the mesohyl tissue of the sponge *H. perlevis*. Cyanobacteria observed in the sponge at time of collection from natural environment (a and b) and from maintenance under controlled conditions, *ex situ*, for 15 days (c and d). In pictures a and c it is possible to observe the existence of cyanocytes. Pictures b and d show the cyanobacteria in more detail, where it is possible to observe the presence of spiral thylakoids.

In respect to cyanobacterial diversity, at genus level, it was possible to identify the presence of OTUs assigned to *Acaryochloris* in sponge samples and *Acaryochloris*, *Synechococcus* and *Phormidium* in seawater. In all samples, OTUs of unidentified cyanobacteria were also present.

Rarefaction analysis of alpha-diversity, through measurements of bacterial richness and diversity in marine sponges and seawater were also quantified. Observed richness and estimated richness (Chao1) are represented in the rarefaction graphs in Figure 4-5. Analysis of Figure 4-5 shows that the amount of observed OTUs was less than the estimated and that the diversity depends very strongly on the sequencing depth. For sponge samples rarefaction curves start to stabilize at around 250 sequences/sample, showing a good depth.

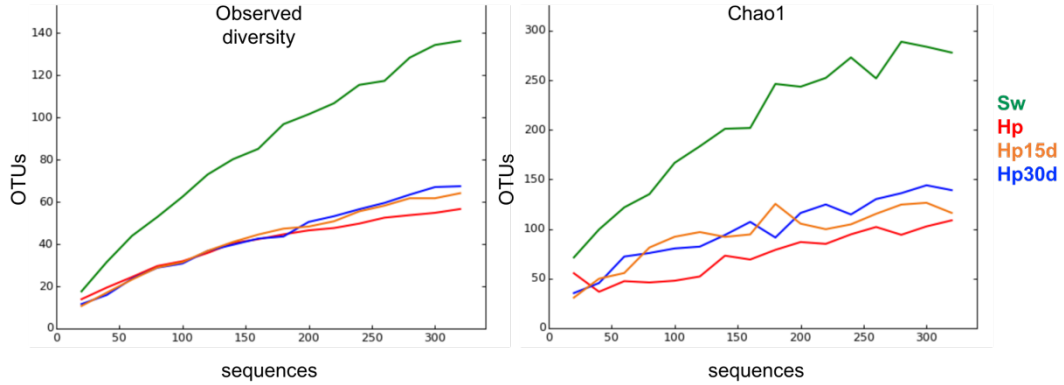

Figure 4-5. Rarefaction curves depicting cumulative community richness: observed diversity and Chao1 (estimated diversity) for the normalized dataset. Sw: seawater (green); Hp: *H. perlevis in situ* (red); Hp15d: *H. perlevis ex situ* for 15days (orange); *H. perlevis ex situ* for 30 days (blue).

Community diversity was determined using Shannon indices, as presented in the rarefaction curves in Figure 4-6. Results show the diversity not to change greatly in accordance to the number of sequences per sample. A higher diversity for seawater, when compared to sponge samples. In between sponge samples, there is not a big difference in diversity.

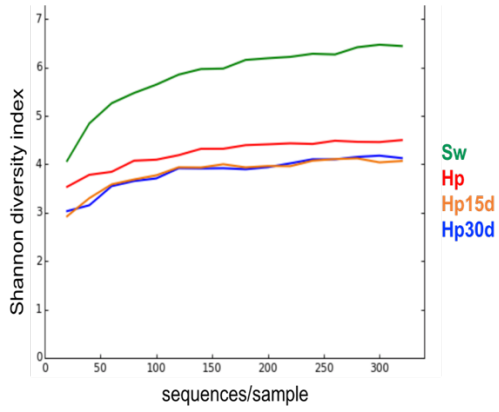

Figure 4-6. Rarefaction curves depicting cumulative community diversity: Shannon diversity index for the normalized dataset. Sw: seawater (green); Hp: *H. perlevis in situ* (red); Hp15d: *H. perlevis ex situ* for 15days (orange); *H. perlevis ex situ* for 30 days (blue).

Beta-diversity allowed the comparison in between samples. Principal coordinates analysis (PCoA) is shown in Figure 4-7 allowing to infer that seawater diversity is much more different from sponge samples than sponge samples in between them. Also, the bacterial community of sponge's changes along the time under controlled conditions.

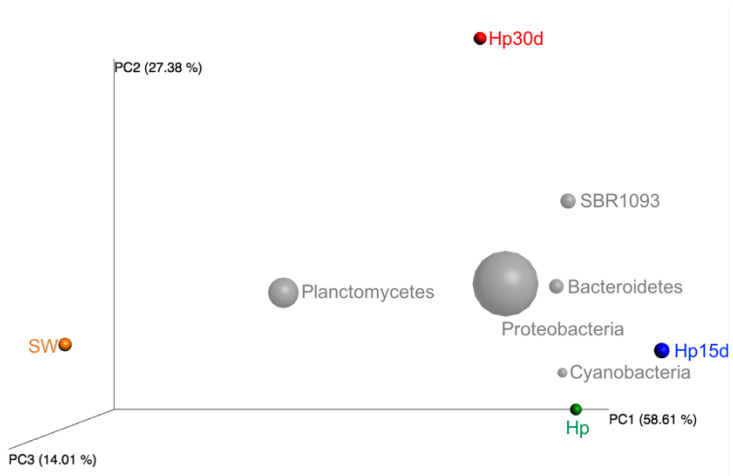

Figure 4-7. Principal coordinates analysis (PCoA) based on weighted UniFrac distance metric of the most common bacterial community profiles at phylotype (OTUs) level. Samples are presented by color: Sw: seawater (green); Hp: *H. perlevis in situ* (red); Hp15d: *H. perlevis ex situ* for 15days (orange); *H. perlevis ex situ* for 30 days (blue). The 5 most dominant bacterial taxa (at phylum level) are shown and respective assigned OTUs. The position of bacterial taxa was determined by correlation of relative abundances and sample categories.

In Figure 4-8, a Venn diagram is shown to elucidate shared and unique OTUs in samples. The diagram shows that only 16 OTUs are shared for all samples, and only 9 for sponges.

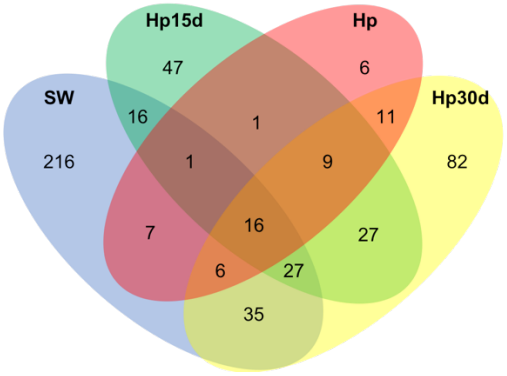

Figure 4-8. Venn diagram showing number of OTUs shared and unique to each sample. Sw: seawater; Hp: *H. perlevis in situ*; Hp15d: *H. perlevis ex situ* for 15days; *H. perlevis ex situ* for 30 days.

Discussion

In the present study we intended to assess how the microbial community associated with a common intertidal marine sponge from the coast of Portugal, would change when translocated to laboratory-controlled conditions. The chosen sponge, *Hymeniacidon perlevis*, has been used previously in different studies to assess the microbial community by our group (Alex et al., 2012, Alex et al., 2013, Regueiras et al., 2017), and has a broad geographical distribution along the Atlantic Ocean, North Sea, Mediterranean Sea (Van Soest et al., 2018), showing the importance of the study of this sponge on a global scale.

Next-generation sequencing (NGS) technologies allowed to deeply understand microbial community richness. Recently, the global sponge microbiome project started assessing microbial communities from sponges, around the globe using standardized protocols (Thomas et al., 2016, Moitinho-Silva et al., 2017). Prevalence of microbial community within marine invertebrates showed that these organisms cannot be studied individually and must be assumed as metaorganisms (Bosch & McFall-Ngai, 2011). Due to all advantages from these associations, symbionts are known to influence both health and functioning of the hosts (Pita et al., 2018).

Here we applied NGS to understand how the microbial community would be affected by translocation of sponge to *ex situ* conditions, and if those changes could affect sponge viability.

First, we unveiled a much different bacterial community between the water column (Sw) and the one in the sponge *in situ* (Hp) (Figure 4-3), with only 7 OTUs shared by both (Figure 4-8). The results here obtained are in accordance to the ones from previous studies (Hentschel et al., 2002, Hentschel et al., 2006, Taylor et al., 2007a, Hentschel et al., 2012, Webster & Taylor, 2012). Webster et al. (2010) found sponge OTUs to be present in seawater at very low abundances. In between sponge samples, a high discrepancy in both OTUs observed and number of sequences was found. PCR-based techniques may influence the results, as specific taxonomic groups can be favoured and disproportionally be amplified, affecting richness estimations in environmental samples (Webster et al., 2010).

From sponge samples, altogether, 21 microbial phyla (or candidate phyla) were identified, suggesting a complex microbial community. Our data revealed that bacterial composition from each sample were different from each other, and through the beta-diversity analysis performed (Figure 4-7), it is possible to see how the community changes from the sponge collected from the natural environment (Hp) and the community after 30 days under controlled conditions (Hp30d). The most dominant sponge-associated phyla were Proteobacteria, Planctomycetes, Cyanobacteria and Bacteroidetes. Alpha- gamma- and delta-proteobacteria were the major classes. Proteobacteria are an important group found in almost all sponge microbial diversity studies (Thomas et al., 2016, Moitinho-Silva et al., 2017), and showed to not change drastically after sponge translocation in our work. Alex and Antunes (2015), using NGS analysed the microbial community associated with different marine intertidal sponges (n=12) from the coast of Portugal, finding also Proteobacteria to be the main phyla present in all sponge species, as well as the presence of both Planctomycetes and Cyanobacteria.

It has been pointed before that *H. perlevis* is a low microbial abundance sponge (LMA) (Alex et al., 2013, Regueiras et al., 2017), meaning that it has a microbial concentration similar to the one present in the water column, and Weigel and Erwin (2016) suggested that intertidal sponges may have a less diverse microbial community due to constrictions from living in a physiological stress area (air exposure and high temperature oscillations). It is known that environmental conditions can affect the microbial community (Morrow et al., 2016, Steinert et al., 2016, Weigel & Erwin, 2017). Disturbing the balance that exists between sponge and symbiotic microorganisms can affect both the sponge and the hosts, changing the production of secondary metabolites or even interfering in sponge survival. Pita et al. (2018) refers that disturbance in the holobiont can lead to three different scenarios: through resistance and resilience the community can stay stable, or microbial community can change so drastically affecting sponge survival, or even can lead to a new acclimations and adaptation to novel environmental conditions.

Under controlled conditions after 30 days *ex situ*, the most important remark was the almost absence of the phylum Cyanobacteria (1 OTU, 3 sequences). The observed trend was confirmed by TEM analysis, where we were unable to detect any cyanobacteria in sponge tissue after that period. In contrast, both the sponges collected from the natural environment (Figure 4-4a-b) and after 15 days under controlled conditions (Figure 4-4c-d), presented a large cyanobacterial community within specialized arqueocytes vacuoles (Rützler, 1990) named cyanocytes. The combination of molecular techniques and microscopic ones has been suggested as a better approach and used in different studies to address cyanobionts composition in sponges (Alex et al., 2012, Bayer et al., 2014).

Photosynthetic bacteria, such as cyanobacteria provide many benefits for the host, such as supplemental nutrition (Wilkinson & Fay, 1979, Wilkinson, 1980) and through production of secondary metabolites can provide defence (Carpenter & Foster, 2002), protection from U.V light (Adams, 2000) and help in substrate competition (Usher et al., 2004, Taylor et al., 2007a). Recently, a detailed review on the sponge-cyanobacteria associations was made by Konstantinou et al. (2018), concluding that these associations are as common in temperate areas, as in tropical ones. The nature of these associations can be affected by physical and environmental aspects (Usher, 2008), and some sponges are unable of surviving without their cyanobionts (Thacker, 2005). Steindler et al. (2007) found the expression from some sponge genes to be related to the presence of cyanobionts, and Morrow et al. (2014) found sponges to be able to survive and even to enhance its growth under new climate scenarios, such as ocean acidification, due to harbouring a significantly higher abundance of *Synechococcus* species, which provide the host with a nutritional benefit. After 30 days sponge viability was compromised, and

the sponge died. We here hypothesized that the changes observed in the cyanobacterial community were, at least in part, responsible for that overcome.

Sponges are important members of benthic communities, with a huge biotechnological potential, and capable of providing more insights on conserved mechanisms of host-microbe relations in basal metazoans. The fact that sponges occur at all geographical aquatic locations, and associations with microbes occur across different species, make them good laboratory models. Assuming the sponge and their microbial community as a metaorganisms and knowing that maintenance under controlled conditions can be challenging and assessing the microbial community, it changes and how it affects sponge survival is the first step towards the use of these organisms as models. Here, we provided a first assessment on bacterial community changes and we hypothesized that sponge viability was compromised by the loss of cyanobionts. Further investigations must be made in order to confirm the results here presented.

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Supplement material

QIIME script

Split_libraries.py

denoise_wrapper.py

inflate_denoiser_output.py

extract_seqs_by_sample_id.

pick_otus.py

pick_rep_set.py

identify_chimeric_seqs.py

filter_fasta.py

assign_taxonomy.py

make_otu_table.py

biom convert

biom summarize-table

sort_otu_table.py

split_otu_table_by_taxonomy.py

summarize_taxa.py

make_otu_heatmap.py

plot_taxa_summary.py

multiple_rarefactions.py

align_seqs.py

filter_alignment.py

make_phylogeny.py

alpha_diversity.py

collate_alpha.py

make_rarefaction_plots.py

beta_diversity_through_plots.py

jackknifed_beta_diversity.py

make_bootstrapped_tree.py

dissimilarity_mtx_stats.py

make_emperor.py

dissimilarity_mtx_stats.py

make_distance_boxplots.py

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Chapter 5. Differential toxicity of Cyanobacteria isolated from marine sponges towards echinoderms and crustaceans

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Differential Toxicity of Cyanobacteria Isolated from Marine Sponges towards Echinoderms and Crustaceans

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Differential toxicity of Cyanobacteria isolated from marine sponges towards echinoderms and crustaceans

Abstract

Marine sponges and cyanobacteria have a long history of co-evolution with documented genome adaptations in cyanobionts. Both organisms are known to produce a wide variety of natural compounds, with only scarce information about novel natural compounds produced by cyanobionts. In the present study, we aimed to address their toxicological potential, isolating cyanobacteria (n=12) from different sponge species from the coast of Portugal (mainland, Azores and Madeira Islands). After large-scale growth, we obtained both organic and aqueous extracts to perform a series of ecologically-relevant bioassays. In the acute toxicity assay using nauplii of *Artemia salina,* only organic extracts showed lethality, especially in picocyanobacterial strains. In the bioassay with *Paracentrotus lividus*, both organic and aqueous extracts produced embryogenic toxicity (respectively 58% and 36%), pointing to the presence of compounds that interfere with growth factors on cells. No development of pluteus larvae was observed for the organic extract of the strain *Chroococcales* 6MA13ti, indicating the presence of compounds that affect skeleton formation. In the hemolytic assay, none of the extracts induced red blood cells lysis. Picocyanobacterial strains showed to be the ones with most potential. Organic extracts, especially from picoplanktonic strains, proved to be the most promising for future bioassay-guided fractionation and compounds isolation. This approach allows us to clarify the compounds extracted from the cyanobacteria into effect categories and bioactivity profiles.

Keywords

Marine cyanobacteria; cyanotoxins; marine sponges; secondary metabolites; marine natural compounds; bioassays; *Artemia salina*; *Paracentrotus lividus*; hemolytic essay

Key Contribution

The present work shows for the use of marine sponges as a source for harvesting cyanobacteria. Being adapt to life inside sponges, these cyanobacteria can prove to have novel compounds produced from their secondary metabolism.

Introduction

Cyanobacteria are photosynthetic prokaryotes, with a high morphological, physiological and metabolic diversity, with fossil records dating back to 3.5 billion years ago (Adams & Duggan, 1999). Secondary metabolite production was essential for their survival allowing for adaptation to several environmental conditions such as variations in temperature, pH, salinity, UV radiation among others.

Climate change and eutrophication increased the occurrence and frequency of cyanobacterial blooms in water bodies, posing human and animals' health risks due to toxin production. Apart from toxin production, these secondary metabolites have also been shown to be a source of compounds of interest in different industries, such as pharmaceutical, cosmetics, agriculture, energy, etc. It is estimated that only in the last decade more than 400 new natural compounds were extracted from marine cyanobacteria (Mi et al., 2017). Coastal water blooms pose another health risk concerning cyanobacterial toxins, as many of them are able to accumulate in both vertebrates and invertebrates (Buratti et al., 2017).

Assessing marine cyanobacterial diversity on the Portuguese coast has already been the focus of various studies (e.g.(Brito et al., 2012, Brito et al., 2017)), with *Cyanobium*, *Leptolyngbya* and *Pseudanabaena* as the most abundant genera among isolates (Brito et al., 2012). Isolated strains from the coast of Portugal were found to be a source of bioactive compounds, both with toxicological and/or pharmaceutical interest (Martins et al., 2005, Martins et al., 2007, Martins et al., 2008, Frazão et al., 2010, Leão et al., 2013, Costa et al., 2014, Costa et al., 2015, Afonso et al., 2016). Also, Brito et al. (2015) evaluated the potential to produce secondary metabolites for some strains through molecular methods.

In marine environments cyanobacteria are known to form associations with a variety of invertebrates, such as sponges (Phylum Porifera). Sponges are filter-feeders, capable of filtering thousands of litters of water per day. During this process, some filtered microorganisms can become part of the sponge microbiota, which diversity can reach up to 4 orders of magnitude, when compared to the one from water column (Hentschel et

al., 2006). In temperate ecosystems, it is estimated that 45-60% of sponges to have cyanobacterial symbionts (cyanobionts) (Lemloh et al., 2009), and are capable to cover up to 50% of the sponge cell volume (Rützler, 1990). As they are able to concentrate microorganisms, sponges can be used as a source for cyanobacteria harvesting as already stated by Regueiras et al. (2017). Sponges are a huge source of bioactive compounds (Blunt et al., 2010), most of them known to be produced by their symbiotic microorganisms (Hentschel et al., 2006). Actinobacteria, Cyanobacteria, Firmicutes and Proteobacteria (alpha and gamma classes) are the main phyla producing secondary metabolites in sponges (Thomas et al., 2010a).

Both coccoid and filamentous cyanobacteria have been described in sponges. Recently, Konstantinou et al. (2018) made a review on the diversity of both sponge species harboring cyanobacteria, and cyanobacterial diversity. In Portugal, *Xenococcus*-like and *Acaryochloris* sp. were reported from the intertidal marine sponge *Hymeniacidon perlevis* (Alex et al., 2012, Alex & Antunes, 2015). Regueiras et al. (2017) were also able to identify cyanobacteria belonging to the genera *Synechococcus*, *Cyanobium*, *Synechocystis*, *Nodosilinea*, *Pseudanabaena*, *Phormidesmis*, *Acaryochloris* and *Prochlorococcus* associated with the same marine sponge.

Due to a long evolutionary history of both cyanobacteria and marine sponges, co-evolution has already been documented, with some cyanobacteria being passed to new sponge generations through vertical transmission (from sponge to offspring through reproductive cells) (Usher et al., 2001). The study of genomes from the symbiotic cyanobacteria "Ca. *Synechococcus spongiarum*" and its comparison with the genome of free-living ones, found adaptations to life inside sponges and the presence of different adaptations in different phylotypes (Gao et al., 2014, Burgsdorf et al., 2015). These adaptations may also lead to the production of novel and unique natural compounds.

Bioassay-guided fractionation is a successful strategy in the isolation and discovery of novel compounds (Papendorf et al., 1998, Luesch et al., 1999, Luesch et al., 2000, Mundt et al., 2001, Han et al., 2006). To address toxin production several assays can be used. The use of the brine shrimp *Artemia salina* has ecological relevance in marine ecosystems, as these organisms are a representation of the zooplankton community and vital on the ecology of seashores (Martins et al., 2007). For embryogenesis studies, the use of echinoids, such as the sea urchin *Paracentrotus lividus* is very common. They occupy an important phylogenetic position (deuterostomes) when compared to other invertebrates. P. *lividus* are also common among the Portuguese seashore and key elements on their habitats, capable of producing a great amount of eggs feasible to be

fertilized in seawater, and to develop optically clear embryos (Lopes et al., 2010). Apart from these common assays, less is known on hemolytic toxins from cyanobacteria. Cyanobacterial toxins are able to accumulate in marine vertebrate and invertebrates (Engström-Öst et al., 2002, Ferrão-Filho et al., 2002), posing risks for mammals, showing the importance of the use of such assays.

The present study aims to do a preliminary assessment on the cyanotoxin potential of marine cyanobacteria isolated from marine sponges. Most studies isolate marine cyanobacteria through filtration of large volumes of water, or by scratching coastal surfaces. In the present study we aimed to isolate cyanobacteria from marine sponges of the coast of Portugal, as they are able to concentrate microorganisms, allowing to obtain some cyanobacteria that can be present in seawater in amounts under detection.

Materials and methods

Cyanobacterial strains selection and biomass production

Cyanobacterial strains used in this study were previously isolated from marine sponges. Marine sponges were collected both from seashore rocks and by scuba diving and a small fraction of the sponge tissue was collected in flaks with ambient seawater. Figure 5-1 shows sampling locations, being all intertidal sites, with exception from the one in Madeira Island, Caniçal, (sponges collected through scuba diving). When collected from intertidal areas, beaches were chosen with a combination of sand and rocks. Sponges substratum were rocks or sand. Preparation of sponge samples and cyanobacterial isolation and characterization was done according to Regueiras et al. (2017). Summarizing, sponges were cleaned of debris and 1 mm of the sponge surface was discarded, using a sterile razor to avoid cultivation of superficial bacteria. Small fragments of the sponge body (<0.5 cm^3) were placed in 2 different culture media, Z8 liquid media (Kótai, 1972), supplemented with 30 g l^{-1} of NaCl and MN liquid medium (Rippka, 1988). Both culture media were supplemented with vitamin B12 and cyclohexamide (Rippka, 1988). After growth, through micromanipulation techniques, as described by Rippka (1988), a single cell or filament of cyanobacteria were transfer to new liquid medium, until achievement of unicyanobacterial, non-axenic cultures.

The selection of cyanobacterial strains was based on growth performance rates and cyanobacterial diversity. Morphological identification followed the criteria of Komárek and Anagnostinis (Komárek & Anagnostidis, 2005, Komárek, 2008, Komárek, 2013), the Bergey's manual of systematic bacteriology (Castenholz et al., 2001) and Komárek et al. (2014). Strains are deposited in the LEGE Culture Collection (Ramos *et al*., 2018). The

twelve strains selected (Table 5-1) were cultured and up-scaled under laboratory conditions at 25°C, light/dark cycle of 14/10 h and light intensity of approximately 25 × 10^{-6} E/$m^{-2}s^{-1}$. After 60 to 90 days of growth, the cyanobacterial biomass produced was collected (through centrifugation or filtration with a 20 µm pore net), frozen at -20 ºC and freeze dried. Lyophilized material was kept at -20 ºC.

Table 5-1. Cyanobacterial strains selected for the present study, with information about the marine sponge it was isolated from and collection site.

Cyanobacterial strain	**Sponge species**	**Collection site**
Synechococcus sp. LEGE11381	*Polymastia* sp.	Memória
Synechocystis sp. 44B13pa	*Polymastia* agglutinans	São Roque, Azores
cf. *Phormidesmis* sp. LEGE10370	*Hymeniacidon perlevis*	Memória
Unidentified filamentous *Synechococcales* LEGE11384	*Phorbas plumosus*	Memória
Phormidium sp. 25J12tp	*Tedania pilarriosae*	Memória
Nodosilinea cf. *nodulosa* LEGE10376	*Hymeniacidon perlevis*	Porto Côvo
Chroococcales 6MA13ti	*Tedania ignis*	São Roque, Azores
Leptolyngbya sp. 31H12hpa	*Halichondria panicea*	Memória
Cyanobacterium 34C12sp	Unidentified sponge	Caniçal, Madeira
Cyanobium sp. LEGE10375	*Hymeniacidon perlevis*	Memória
Pseudanabaena aff. *curta* 12C10hp	*Hymeniacidon perlevis*	Memória
Leptolyngbya sp. 31B12op	*Ophlitaspongia papila*	Memória

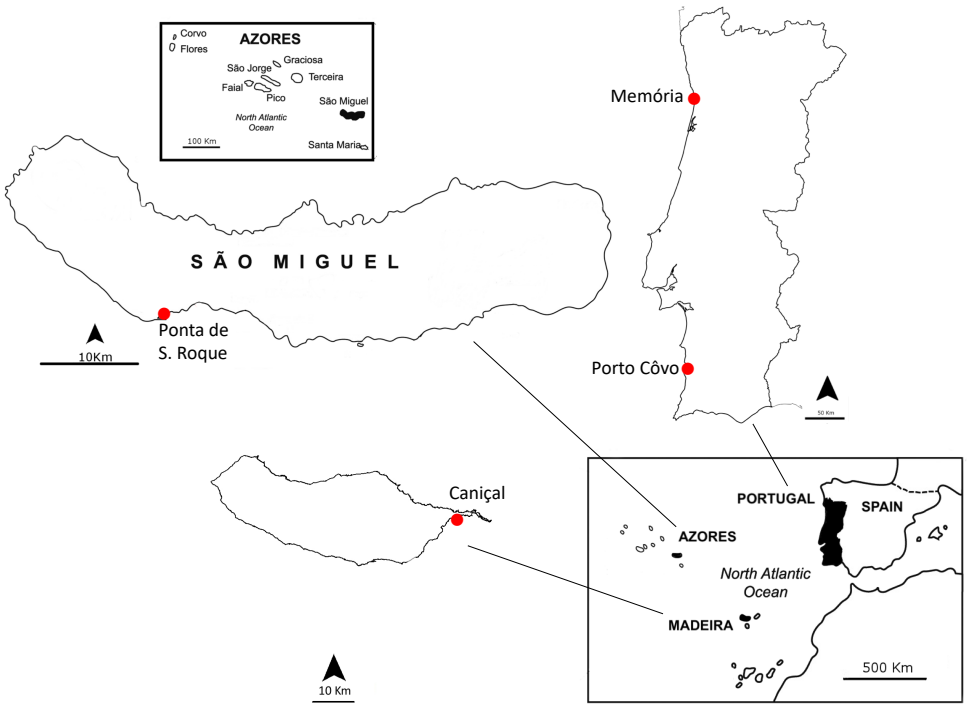

Figure 5-1. Sampling locations. Two sampling locations were in Portugal mainland: Memória (N 41º13'52.27", W 8º43'18.34") and Porto Côvo (N 37º52'3.04'', W 8º47'37.19"). One was in Madeira Island: Caniçal (N 32º44'20.08'', W 16º44'17.55''). And the other in São Miguel Island, Azores: São Roque (N 37º45'15,35'', W 25º38'31.60'').

Preparation of cyanobacterial extracts

The freeze dried biomass from each cyanobacterial strain was repeatedly extracted with a warm (<40 °C) mixture of dichloromethane and methanol (CH2Cl2:MeOH) (2:1) (P.A. Sigma, USA). Afterwards, the solvents were removed in vacuo and/or under a N_2 stream. Following the organic extraction, the remaining biomass was subjected to aqueous extraction (ultra-pure water), decanted and centrifuged at 4600 rpm for 15 min. The resulting supernatant was freeze-dried, weighed and stored at −20 °C. Just before the tests, organic extracts were dissolved (30 mg mL^{-1}) in dimethyl-sulfoxide (DMSO) and aqueous extracts in ultra-pure water.

Bioassays

Acute toxicity assay using nauplii of *Artemia salina*

In the acute toxicity assay, the nauplii of the crustacean *Artemia salina* were used. The dried cysts (JBL Novotemia, Germany) hatched after 48h in 35 g/L filtered seawater, at 25 °C, under conditions of continuous illumination and aeration. Toxicity was screened

in a 96-well polystyrene plate, with 10-15 nauplii per well and 200 μL of organic or aqueous extract. The negative controls were filtered seawater and filtered seawater with 0.1% DMSO. As for the positive control was used potassium dichromate at a concentration of 8 μg/mL. Four replicates were made for each treatment. The plates were covered with Parafilm to prevent water loss and then incubated at 25 °C, for 48 h in darkness. Dead larvae were counted in each well on an inverted microscope at 24 h and 48 h. Before determining the total number of larvae, organisms were fixed with a few drops of Lugol's solution. Mortality was calculated through percentage as described by Martins et al. (2007).

Embryo – larval acute toxicity assay with *Paracentrotus lividus*

For the embryo-larval acute toxicity assay, sea urchins *Paracentrotus lividus* were captured in the intertidal rocky shore, during low tide in Praia da Memória, Matosinhos, Portugal and immediately transported to the laboratory, in natural sea water and under refrigeration. The protocol employed was the one described by Fernández and Beiras (2001). Briefly, a couple of specimens were dissected, and gametes were collected with a pipette directly from the gonads. The optimal condition from gametes (spherical eggs and mobile sperm) was granted through careful observation under the optical microscope. Eggs were transferred to a 100 mL measuring cylinder containing natural seawater filtered through a 0.45 μm pore filter. A few microliters of sperm were added to the eggs suspension and then carefully stirred to allow fertilization. Fertilized eggs were counted in four 10 μL aliquots in order to determine the fertilization success and egg density. In a 24-well plate, a concentration of 20 fertilized eggs per mL of solution were exposed to organic and aqueous extracts, during 48 h at 20 °C, in darkness. Test solutions consisted of 2.5 mL of each cyanobacterial extract; two negative controls were used, one with only filtered seawater and the other with 0.1% DMSO; as positive control was used potassium dichromate in a concentration of 4 μg/mL. Four replicates were made for each treatment. After 48 h of incubation, the solutions were fixed with 40% formalin. Results were evaluated through percentage of pluteus larvae (embryogenic success) and larval length (larval growth) (Martins et al., 2007).

Hemolytic assay

For the hemolytic assay, mice blood, stabilized with heparin, was provided by IBMC Bioterium, from healthy specimens without need to sacrifice the animals. The protocol used was an adaptation of the ones described by Rangel et al. (1997) and Slowing et al. (2009). Summarizing, the erythrocytes solution was diluted with 30 volumes of a saline solution (0.85% NaCl with 10 mM $CaCl_2$) and centrifuged at 1100 g for 5 minutes,

discarding the supernatant and then washed three times with the same solution followed by centrifugations (1100 g for 5 min). After the final wash, the cells were diluted to a final concentration of 1% in sterile PBS solution. The assay was performed with 100 μL of each extract mixed with equal volume of erythrocytes suspension, using three replicates per treatment. For the negative and positive controls were used PBS and 0.1% Triton100, respectively. Eppendorfs with the mixtures were incubated for 2 hours, at a temperature of 37 ºC, with slow agitation. After that period, the mixtures were centrifuged at 4000 g for 1 minute at 4 ºC. The supernatants were transferred to a 96 well plate. Hemoglobin content was evaluated spectrophotometrically at 540 nm (Rangel et al., 1997).

$$Hemolytic\ activity = \frac{Abs_{\ sample} - Abs_{\ negative\ control}}{Abs_{\ positive\ control} - Abs_{\ negative\ control}} \times 100\%$$

Statistical analysis

Data collected during the bioassays were analyzed using a one-way analysis of variance (ANOVA), followed by a multi-comparisons Dunnett test ($p < 0.05$). The software IBM SPSS Statistics 24 (Version 24.0.0.0 edition 64-bit, IBM Corporation, NY, USA, 2016) was used for statistical analysis.

Results

Acute toxicity assay using nauplii of *Artemia salina*

In the bioassay to assess mortality in *Artemia salina* nauplii (Figure 5-2), aqueous extracts from the selected cyanobacterial strains did not exhibit statistically significant differences, when compared against the control. However, for the organic extracts, toxicity was found after 48h of exposure. Cyanobacterial strains *Synechococcus* sp. LEGE11381 ($F=68.80$, $p<0.000$), *Synechocystis* sp. 44B13pa ($F=21.82$, $p<0.048$), unidentified filamentous *Synechococcales* LEGE11384 ($F=24.74$, $p<0.018$), *Chroococcales* 6MA13ti ($F=86.73$, $p<0.000$) and *Cyanobium* sp. LEGE10375 ($F=43.50$, $p<0.000$) presented statistically significant differences when compared against the negative control.

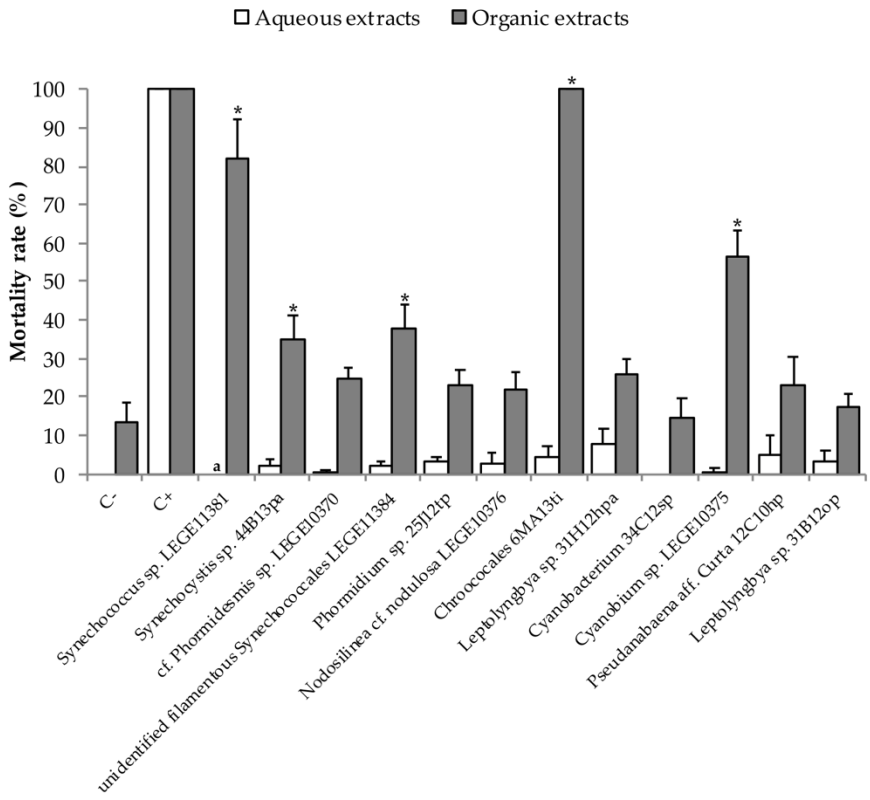

Figure 5-2. Mortality rate for the *Artemia salina* bioassay, after 48h of exposure, with organic and aqueous extracts. The *Synechococcus* sp. LEGE11381 strain was not present in the aqueous extract. Controls used included filtered seawater with 0.1% DMSO for negative control and potassium dichromate ($8\ \mu g/ml$) for positive control. *Statistically significant differences between extract and control.

Embryo – larval acute toxicity assay with *Paracentrotus lividus*

The viability of the sea urchin assay was measured through the analysis of embryogenic success, i.e. the ability of the fertilized egg to reach the stage of pluteus larvae, and through the growth of pluteus larvae in length (Figure 5-3). Development arrest indicates that no normal pluteus larvae were produced. The results gathered after 48h of incubation with cyanobacterial extracts revealed that in the control, 67.5 ± 6.1% of the sea urchin fertilized eggs developed to normal pluteus larvae, with an average length of 330,0 ± 18,8 µm. Figure 5-4 shows significant difference in the embryogenic development, at $p<0.05$, for the organic extract of the following strains: *Synechococcus* sp. LEGE11381 ($F=-62.78$, $p<0.000$), *Synechocystis* sp. 44B13pa ($F=-41.80$, $p< 0.000$), unidentified filamentous *Synechococcales* LEGE11384 ($F=-36.05$, $p<0.000$), *Phormidium* sp. 25J12tp ($F=-27.22$, $p<0.010$), *Leptolyngbya* sp. 31H12hpa ($F=67.48$, $p<0.048$) and *Cyanobium* sp. LEGE10375 ($F=-52.38$, $p<0.000$). The organic extract of the strain *Chroococcales* 6MA13ti caused development arrest with none of the larvae

reaching the stage of viable pluteus. Amongst the aqueous extracts, unidentified filamentous *Synechococcales* LEGE11384 (F=-41.75, p<0.001), *Phormidium* sp. 25J12tp (F=-28.75, p<0.033), *Chroococcales* 6MA13pi (F=-30.00, p<0.024) and *Cyanobacterium* 34C12sp (F=-39.25, p<0.002) strains presented significant embryogenic effect. Regarding the results from the positive control, only embryos on gastrula stage were found.

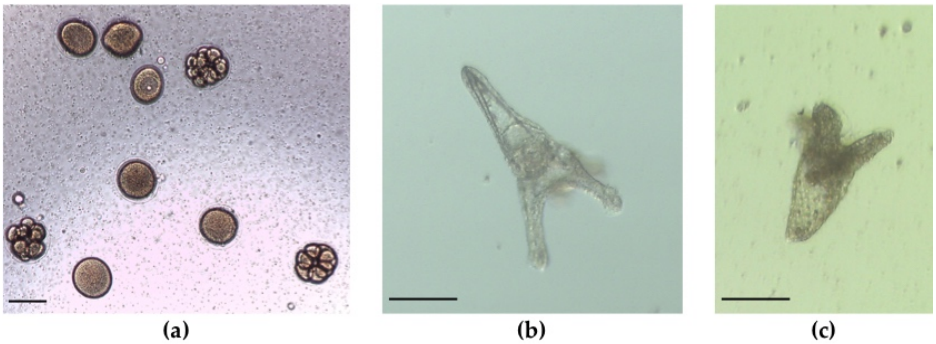

Figure 5-3. Effects of marine cyanobacterial extracts on embryogenesis of the sea urchin *Paracentrotus lividus*. (a) Fertilized sea urchin eggs; (b) Normal pluteus larvae resulting from control treatment and (c) Abnormally developed larvae resulting from treatments with cyanobacterial extracts. Scale bar: 100 μm.

Regarding larval growth data, homogeneity in larval length was evidenced in the aqueous extracts at p<0.05 [F (11, 36) =1.039, p<0.434)] (Figure 5-5). However, differences in larval length were found in organic extracts. These differences were more

significant in *Synechococcus* sp. LEGE11381 (246.2 ± 11.5μm, p<0.001) and Cyanobium sp. LEGE10375 (325.7 ± 9.7μm, p<0.000).

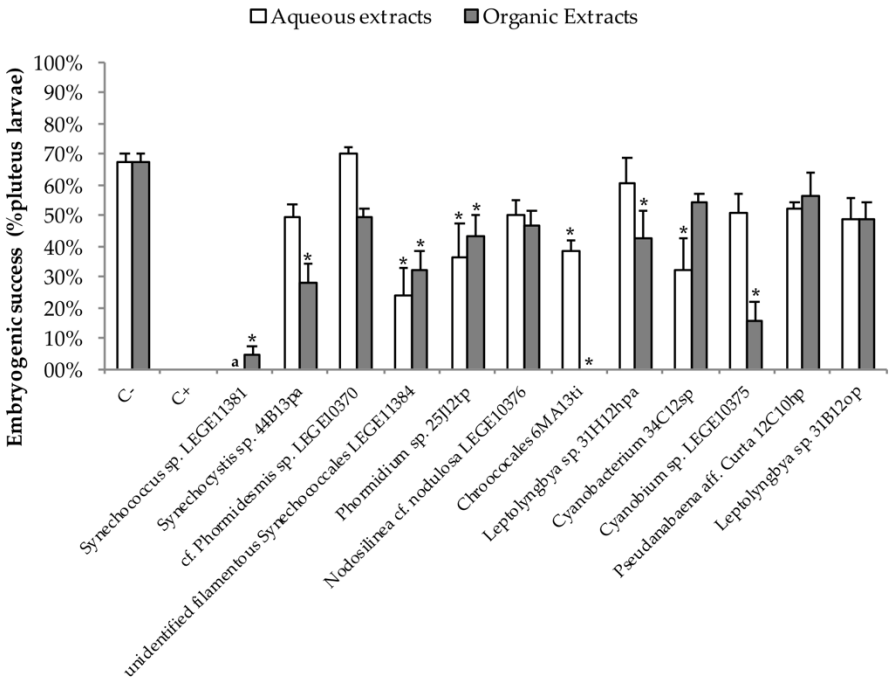

Figure 5-4. Embryogenic success from the aqueous and organic extracts of the cyanobacterial strains represented by percentage of pluteus larvae developed. For the controls it was used filtered seawater with 0.1% DMSO (negative) and potassium dichromate at 4μg/ml (positive). *Statistically significant differences between extract and control.

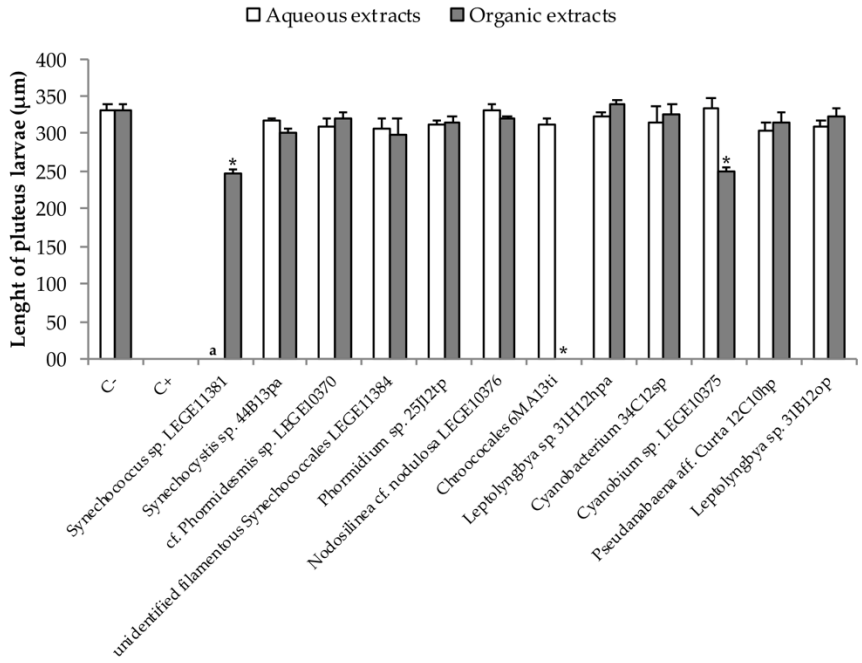

Figure 5-5. Larval growth from the organic extracts of the cyanobacterial strains. For the controls it was used filtered seawater with 0.1% DMSO (negative) and potassium dichromate at 4μg/ml (positive). *Statistically significant differences between extract and control.

Hemolytic assay

The hemolytic activity registered during the assay was below 10%, with the highest value obtained being 7% of activity by the strain *Chroococcales* 6MA13ti, in the organic extract. The remaining strains and extracts did not present significant interference with the haemoglobin content.

Discussion

To date, most studies exploring the bioactivity of marine cyanobacteria have been focusing on free-living forms. Cyanobacteria can live in association with a variety of marine invertebrates, such as sponges, and it is known that cyanobacteria can affect the biosynthesis of compounds from the host (Ridley et al., 2005) and that symbionts have specific adaptations in their genome (Gao et al., 2014, Burgsdorf et al., 2015). The biological potential of associated and/or symbiotic cyanobacteria is still mostly unexplored. In the present study, twelve marine cyanobacterial strains were isolated from sponges of the Portuguese coast. These strains were submitted to a bioassay-guided ecologically-relevant bioassays in order to assess the production of secondary metabolites with toxicological or pharmaceutical interest.

Artemia spp. is known for its ability to adapt to different environmental conditions, making it a crucial test organism in ecotoxicology (Nunes et al., 2006). Results from the bioassay with the brine shrimp *Artemia salina* nauplii showed that the aqueous extracts of the tested cyanobacterial strains did not display acute toxicity towards the nauplii. The organic extracts of *Synechococcus* sp. LEGE11381, *Synechocystis* sp. 44B13pa, unidentified filamentous *Synechococcales* LEGE11384, *Chroococcales* 6MA13ti and *Cyanobium* sp. LEGE10375 cyanobacterial strains proved to be the most toxic to this crustacean species. In contrast with our results, most previous studies with cyanobacteria from the coast of Portugal found aqueous extracts to be more toxic. For example, Leão et al. (2013) reported lethality towards *A. salina*, in aqueous extracts in free-living forms from *Nodosilinea*, *Leptolyngbya* and *Pseudanabaena* genera strains. Also, Frazão et al. (2010) found aqueous extracts of the genera *Cyanobium*, *Synechococcus*, *Leptolyngbya*, *Oscillatoria* and *Phormidium* more toxic than organic ones. In brackish waters Lopes et al. (2010) also found aqueous extracts more toxic, and organic extracts did not induced more than 7% of mortality. Pagliara and Caroppo (2011) found aqueous extracts of *Leptolyngbya* sp. and *Synechococcus* sp. isolated from the marine sponge *Petrosia ficiformis* to cause acute toxicity. The higher values of mortality here observed were all in picocyanobacterial strains. Costa et al. (2015) already reported

the potential of these cyanobacteria as a source for novel metabolites. In the present work, toxicity was only found after 48h. The present results may infer that cyanobacteria associated with marine sponges may produce different metabolites (present in organic extracts) with low ecotoxicity, and therefore their future potential for drug discovery.

In the bioassay with sea urchin *Paracentrotus lividus*, embryogenic toxicity occurred in 58% of the organic extracts and in 36% of the aqueous extracts tested. The unidentified filamentous *Synechococcales* LEGE11384, *Phormidium* sp. 25J12tp, *Chroococcales* 6MA13ti cyanobacterial strains demonstrated embryogenic toxicity in both extracts, which may lead us to infer that, for the same cyanobacterial strain, chemically different bioactive compounds are produced, having the same effect on embryogenic activity of the sea urchin. Although the *Synechocystis* sp. 44B13pa, unidentified filamentous *Synechococcales* LEGE11384, *Phormidium* sp. 25J12tp, *Leptolyngbya* sp. 31H12hpa, *Chroococcales* 6MA13pi and *Cyanobacterium* 34C12sp cyanobacterial strains have demonstrated to be embryotoxic, no alteration on larval length was observed. This may suggest that the toxicity showed by these cyanobacterial strains only affected the early life stages of the sea urchin embryos development, providing strong evidence for the presence of compounds which interfere with growth factors on cells (Martins et al., 2007). The organic extracts of *Synechococcus* sp. LEGE11381 and *Cyanobium* sp. LEGE10375 exhibited interference with the embryogenic development and also with the larval growth. From all the extracts tested, the organic extract from *Chroococcales* 6MA13ti seemed to have the most potent effect on *P. lividus* larvae since it did not allow a normal development of any pluteus larvae. *Cyanobium* sp. organic extracts have already showed to decrease *P. lividus* larvae length (Costa et al., 2015). Lopes et al. (2010) found organic extracts from brackish waters to be more toxic to *P. lividus*, which is in accordance to our results. The inhibition of larval morphogenesis, here observed, point to the presence of compounds that affect skeleton formation.

Hemolytic activity has already been documented in strains of *Synechocystis* (Sakiyama et al., 2006), *Anabaena* (Wang et al., 2005) and *Synechococcus* and *Leptolyngbya* (Pagliara & Caroppo, 2011). From the hemolytic assay, results showed that in neither organic nor aqueous extracts analyzed, the lysis of the red mammalian blood cells was induced. As stated by Pagliara and Caroppo (2011), hemolytic toxins are not common among cyanobacteria.

The identification of new sources of bioactive compounds are a crucial step towards natural drug discovery. The present study aimed to assess a preliminary cyanotoxicological potential from twelve marine cyanobacteria isolated from sponges of

the Portuguese coast. Eight cyanobacterial strains have showed a promising potential on the performed ecologically-relevant bioassays (*Synechococcus* sp. LEGE11381, *Synechocystis* sp. 44B13pa; Unidentified filamentous *Synechococcales* LEGE11384; *Phormidium* sp. 25J12tp; *Chroococcales* 6MA13ti; *Leptolyngbya* sp. 31H12hpa; Cyanobacterium 34C12sp; *Cyanobium* sp. LEGE10375). Furthermore, the concentrations of the extracts here used (30 μg mL−1) are an ecological relevant concentration. This emphasizes the premise that sponges can harbor microorganisms with toxicological interest and that these invertebrates can and should be used in order to isolate new cyanobacteria. The extracts with the most promising bioactivity should be further fractionated to identify with more detail the bioactive compounds. Chemical elucidation should be performed once the purest compounds are achieved.

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Author Contributions

A.R. and V.V. conceived the conceptualization. A.R., S.P. and M.S.C. did the experimental work. Analysis of the data was done by A.R. and S.P. as well as the writing of the original draft. The review and editing of the writing was done by A.R. and V.V.

Conflicts of Interest

The authors declare no conflicts of interest

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Chapter 6. General Discussion

In this thesis, in order to address the various issues, a total of 41 sampling trips (table 6-1) were made, collecting a total of 218 specimens. The majority of the collection effort was made on the northern coast of Portugal (mainland). Sampling effort during the present thesis

Table 6-1. Sampling effort during the present thesis

Geographical locations		Number of sampling trips	
		intertidal	Subtidal (scuba-diving)
Western coast of Portugal	North	20	3
	Centre	3	0
	South	4	0
S. Miguel, Azores		1	3
Madeira Island		2	2

The western Atlantic shore line is a known diversity hotspot for marine invertebrates (Leal et al., 2012), and sponges have been recognized as important members of the ecosystem, both in terms of biomass and species richness, playing significant roles in ecosystem functioning (Xavier & van Soest, 2012) due to being filter feeders.

Due to a lack of information available on sponge diversity from the coast of Portugal, especially the diversity present on intertidal areas, a major effort was done on the identification of these organisms, as presented in Chapter 2. Also, prior to this identification, a review of the available literature from sponges' diversity was made, and here presented in Appendix 1. Most of the information about intertidal sponge diversity comes from the work of Lopes (1989), from her PhD thesis. For the identification of sponges a multidisciplinary approach was employed collecting information about sponges' natural habitat, morphological characters and also molecular information using CO1 as the molecular marker. Overall, the combination of the data collected, together with the one from literature allowed to make for the first time an updated list of intertidal sponges from the western coast of Portugal, with references not only on the Class Demospongiae, but also Calcarea. Praia da Memória, located on the northern part of Portugal harboured a huge diversity of Demosponges. On total, 5 calcarean species and 27 belonging to Demospongiae were identified, 12 of them for the first time in intertidal locations and 11 from the western coast of Portugal.

To improve public awareness on these common intertidal invertebrates, a simple form of divulgation strategy was outlined, through the written of a booklet, a brochure and also a poster, presenting the most common sponge species of the intertidal area. This information is displayed in Appendix II. This information is already well-documented for almost all other common marine invertebrates presented along the Portuguese seashore.

Sponge identification and assessing its diversity showed to be an important first step for other studies. From the collection effort, *Hymeniacidon perlevis* showed to be the most common sponge, proving to be a good candidate for further studies, as the ones presented in chapters 3 and 4.

Sponges are known to harbour a huge diversity of microorganisms. Photosynthetic bacteria, such as cyanobacteria provide many benefits for the host, such as supplemental nutrition (Wilkinson & Fay, 1979, Wilkinson, 1980) and through production of secondary metabolites can provide defence (Carpenter & Foster, 2002), protection from U.V light (Adams, 2000) and help in substrate competition (Usher et al., 2004, Taylor et al., 2007a). In intertidal areas, sponges are prone to air exposure, leading to fluctuations in temperature and irradiance, and lack of filter feeding opportunities (Steindler et al., 2002). During these conditions, the photosymbionts play an important role, providing the sponge hosts with nutrient uptake for their survival and the production of potential UV-screening substances (Steindler et al., 2002).

In chapter 3, using again a multidisciplinary approach (culture dependent and molecular approaches) an assessment of the cyanobacterial diversity associated with the marine sponge *H. perlevis* was made. For that, we isolated cyanobacteria from sponge tissue and cultivate it, and also made a molecular analysis of both the isolated strains, and data collected from DGGE analysis from sponge tissue. Through DGGE, only coccoid cyanobacteria were detected. The absence of a band in the DGGE could mean that the organism was present, but in an amount below the detection limit of the method, explaining why filamentous cyanobacteria were not detected through this method. Employing a relatively inexpensive technique (DGGE) to profile the microbial diversity, provided a first glimpse of the cyanobacterial community, allowing visualization and monitoring of changes directly from the banding patterns. This method, when used for a long period can also allow differentiation between transient and permanent communities (Hentschel et al., 2003). Isolated cyanobacteria showed similarity to already isolated free-living strains. Also, due to the negligible amount of some cyanobacteria in seawater, they could easily be missed when isolating and culturing, and hence their bioactive potential would remain unexploited. Once again, the use of a multidisciplinary approach proved to be complementary to each other. The results here presented, in my point of view show that sponges could be used as a natural filtration and concentration mechanism to obtain new cyanobacterial strains with pharmaceutical potential.

DGGE banding pattern analysis between seawater and sponge samples, suggested the presence of sponge-associated cyanobacterial communities that are distinct from the seawater. The recurrent presence of a cyanobacterial community at different spatial and

temporal scales could be indicative of environmental acquisition of cyanobacteria by the intertidal marine sponge *H. perlevis*. This study (chapter 3) showed, for the first time, the diversity of cyanobacteria associated with marine sponges from the intertidal area of the Portuguese coast (NE Atlantic) using both culture- and molecular-based methods, and the comparison of the sponges' cyanobacterial community to that present in seawater.

The genus *Acaryochloris* was detected through molecular methods both in the work presented in chapter 3 (DGGE banding sequence) and chapter 4 (pyrosequencing), validating *Acaryochloris* as a *H. perlevis*-associated cyanobacterium, which has been reported previously in sponges (Alex et al., 2012). Due to its unique use of far-red light (production of chlorophyll *d*), *Acaryochloris* can live in niches in coastal waters (Murakami et al., 2004), and therefore its presence in intertidal marine sponges is expected, due to their filtration capability. The association of sponges with *Acaryochloris* can be beneficial due to this red-shift chlorophyll. In order to confirm the presence of *Acaryochloris* in *H. perlevis*, in the future, it would be interesting to quantify both chl *a* and chl *d.*

A NGS analysis was done to the sponge *H. perlevis* to assess the bacterial community associated with the sponge and how it changes when sponge is translocated to laboratory conditions. Also, a comparison with the community from seawater was done. This study was presented in chapter 4. Once again, a multidisciplinary approach was performed, combining the results obtained from NGS with a TEM analysis of the sponge tissue.

Results obtained from comparing sponge tissue bacterial community to the one present in seawater were in accordance with the conclusions obtained in chapter 3, showing the community from both to be very different. The diversity of the bacterial community (through number of OTUs) from the tissue of the sponge *in situ*, when compared to the one present in the seawater, was smaller. In Chapter 3, quantification of chlorophyll *a* was also made and the results pointed to the presence of a small photosynthetic community (Erwin & Thacker, 2007) harboured in the sponge *H. perlevis*. Both findings point to *H. perlevis* to be a low microbial abundance sponge. Weigel and Erwin (2016) suggested that intertidal sponges may have a less diverse microbial community due to constrictions from living in a physiological stress area (air exposure and high temperature oscillations). Giles et al. (2013) suggested that LMA sponges may acquire bacteria mainly from ambient seawater. The presence of sponge-associated cyanobacteria from seawater samples (in chapter 3) supports the hypothesis of procurement of symbionts through the environment (horizontal transmission). Furthermore, it indicates the ability of sponge-associated cyanobacteria to survive outside the host tissue (Taylor et al., 2013).

Vertical transmission of cyanobacteria can benefit the offspring by giving them photosynthetic energy before they are able to feed (Lemloh et al., 2009), enhancing its competitive fitness (Oren et al., 2005) but, Maldonado (2007) reported that in some sponges the symbionts are obtained in each new generation from the environment. In the present work, it was intended to obtain larvae from the sponge *H. perlevis* to assess the presence of true cyanobacterial symbionts. Previous studies, using electron microscopy allowed to confirm vertical transmission of cyanobionts to the eggs and larvae of sponges (Usher et al., 2001). According to Stone (1970), the embryos are visible to the naked eye, with a clear breeding period in the warmest part of the year, between July and October. Gaino et al. (2010) found the presence of *H. perlevis* larvae limited to five months, from the end of spring to the late summer. A survey was made to try to find larvae in *H. perlevis*, for 3 years, from April to November. Unfortunately, sponges in a reproductive stage were never found, nor allowing to test the hypothesis here presented of especially a horizontal transmission of cyanobionts.

On chapter 4, from sponge samples, altogether, 21 microbial phyla (or candidate phyla) were identified. Data revealed the bacterial composition from each sample to be different from each other, and beta-diversity analysis showed how the community changed from the sponge collected from the natural environment and the community after 30 days under controlled conditions. The most dominant sponge-associate phyla were Proteobacteria, Planctomycetes, Cyanobacteria and Bacteroidetes. Alpha- gamma- and delta-proteobacteria were the major classes. Proteobacteria showed to not change drastically after sponge translocation.

Disturbing the balance that exists between sponge and symbiotic microorganisms can affect both the sponge and the symbionts, changing the production of secondary metabolites or even interfering in sponge survival. Under controlled conditions after 30 days *ex situ*, the most important remark was the almost absence of the phylum Cyanobacteria (1 OTU, 3 sequences). The observed trend was confirmed by TEM analysis, where we were unable to find any cyanobacteria in sponge tissue at that period. In contrast, both the sponge collected from the natural environment and after 15 days under controlled conditions, presented a large cyanobacterial community within specialized arqueocytes vacuoles (Rützler, 1990) named cyanocytes. The presence of these coccoid cyanobacteria in the cyanocytes point to the presence of a true symbiont. Some sponges are unable of surviving without their cyanobionts (Thacker, 2005). The absence of cyanocytes in sponge tissue after 30 days *ex situ* could have interfered with sponge viability, compromising it and leading to its death. The combination of molecular techniques and microscopic ones proved to be a good and complementary approach.

Sponges are also very important economically, due to the vast production of secondary metabolites, either by their own chemistry or that of their symbionts. Intertidal sponges can also be used as bioindicators for water quality monitoring. Mahaut et al. (2013) used *Hymeniacidon perlevis* as a bioindicator and reported it to have a higher accumulation capacity of contaminants than the mussel *Mytilus edulis* Linnaeus.

Due to the diversity of cyanobacterial strains isolated in the present study from the different marine sponges, an assessment of their toxicological potential was made and presented in chapter 5. Most studies exploring the bioactivity of marine cyanobacteria focus on free-living forms. Cyanobionts can have specific adaptations in their genome (Gao et al., 2014, Burgsdorf et al., 2015) and affect the biosynthesis of compounds from the host (Ridley et al., 2005).

The biological potential of associated and/or symbiotic cyanobacteria is still mostly unexplored. From the bioassay with the brine shrimp *Artemia salina* nauplii, organic extracts of several strains showed to be toxic. On the other hand, the aqueous extracts tested did not display acute toxicity towards the nauplii. This results contrast with the ones made from free-living strains, where aqueous extracts proved to be more toxic (Frazão et al., 2010, Lopes et al., 2010, Leão et al., 2013). Picocyanobacterial strain showed to be more toxic. The present results may infer that cyanobacteria associated with marine sponges may produce different metabolites (present in organic extracts) showing their potential for drug discovery. In the bioassay with sea urchin *Paracentrotus lividus*, organic extracts showed the same trend in both embryogenic development and larval growth. Some strains produced embryogenic toxicity for both extracts, inferring for the presence of a combination of compounds with the same effect on the tested organisms. In one strain, *Chroococcales* 6MA13ti, the organic extract did not allow a normal development of any pluteus larvae. The inhibition of larval morphogenesis, here observed, point to the presence of compounds that affect skeleton formation.

The identification of new sources of bioactive compounds are a crucial step towards natural drug discovery. Eight cyanobacterial strains have showed a promising potential on the performed ecologically-relevant bioassays, emphasizing that sponges can harbour microorganisms with toxicological interest and that these invertebrates can and should be used in order to isolate new cyanobacteria. The extracts with the most promising bioactivity should be further fractionated until chemical elucidation to identify with more detail the bioactive compounds.

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Chapter 7. Conclusions and future perspectives

There was a clear line of work that linked all this thesis, being each chapter not only linked to the others, but also complementary. At each step of the way, each result obtained helped in the decision of what to do next and how the new information could affect and complement the work done so far, raising more insights on the subject.

Here are presented the major conclusions and future perspectives from the present work:

- Sponges (Phylum Porifera) showed to be diverse along the coast of Portugal; Demospongiae are the main class, although members of the class Calcarea were also identified;
- The bacterial community associated with the intertidal marine sponge *H. perlevis* is very diverse and complex and shifts with the environmental conditions, such as translocation of the sponges to controlled conditions can affect the community, interfering on the sponge survival; Although complex, it seems, through analysis of the DGGE banding-pattern that the cyanobacterial community maintains similar along different geographical areas;
- Using more conserved molecular techniques, such as DGGE analysis and sequencing and more advanced ones, as NGS 454-pyrosequencing showed the bacterial and cyanobacterial community to differ from the sponge tissue and water column;
- Through NGS analysis, Proteobacteria OTUs were the most commonly found, followed by Cyanobacteria, Bacteroidetes and Planctomycetes;
- Under controlled conditions, sponges started shifting their bacterial community, with the almost complete loss of cyanobacterial OTUs, also observed by the absence of cyanocytes or cyanobacterial cells through TEM analysis, pointing to the importance of these organisms on sponge survival;
- The impact of cyanobionts on sponge survival should be further investigated;
- The isolated cyanobacterial strains showed phylogenetic similarity to free-living ones pointing to these organisms to be obtained from the water column. Future studies should focus on understanding how cyanobionts are acquired (horizontal and/or vertical transmission);
- Organic extracts from isolated cyanobacterial strains from sponge tissue showed a huge toxicological potential towards echinoderms and crustaceous. Previous studies made using similar free-living strains showed to have aqueous extracts to be more toxic, pointing to novel compounds being produced by these sponge

associated cyanobacteria. A further analysis, fractioning these extracts should be made to uncover the compound, or compounds mixture here present;

- In the present study, when possible, tried to employ multidisciplinary approaches to complement each task. These methods proved complement each result, leading to better understanding each step of the way.

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Chapter 9. Appendix

9.1. Appendix I.

Bibliographic information on the diversity of marine sponges from the Portuguese coast

Here is presented a revision of sponge diversity from the coast of Portugal, already containing the information about sponge diversity obtained from the present study. Table 9-1 shows the diversity within Class Calcarea, Table 9-2 for Class Demospongiae, and Table 9-3 for Class Homoscleromorpha. Apart from the identification of sponge species, it is also presented information on sampling locations and if collection was subtidal or intertidal.

Table 9-1. Bibliographic information on sponge diversity from the coast of Portugal – Class Calcarea

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
Calcaronea	Baerida	Baeriidae Borojevic, Boury-Esnault & Vacelet, 2000	*Leuconia* Grant, 1833	*Leuconia johnstoni* Carter, 1871	Algarve	S	[1]
					Arrábida	S	[2]
				Leuconia nivea (Grant, 1826)	Arrábida	S	[2]
				Leuconia sp.	Arrifana	S	[3]
	Leucosolenida	Grantiidae Dendy, 1893	*Amphiute* Hanitsch, 1894	*Amphiute paulini* Hanitsch, 1894	Sines	S	[4]
			Grantia Fleming, 1828	*Grantia compressa* (Fabricius, 1780)	Aljezur	I	*a*
					VN Mil Fontes	I	*a*
					Apúlia	S	[5]
					Sagres	S	[6]
			Leucandra Haeckel, 1872	*Leucandra aspera* (Schmidt, 1862)	Arrábida	S	[2]
					Sines	S	[4]
				Leucandra bulbosa Hanitsch, 1895	Sines	S	[4]
				Leucandra fistulosa (Johnston, 1842)	Arrábida	S	[2]
				Leucandra gossei (Bowerbank, 1862)	Aljezur	I	*a*
					Apúlia	S	[5]
					Arrábida	S	[2]
					Arrifana	S	[3]
		Sycettidae Dendy, 1893	*Sycon* Risso, 1827	*Sycon ciliatum* (Fabricius, 1780)	VN Mil Fontes	I	*a*
					Arrábida	S	[2]
				Sycon elegans (Bowerbank, 1845)	Arrábida	S	[2]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
				Sycon humboldti Risso, 1827	Apúlia	S	[5]
				Sycon raphanus Schmidt, 1862	Arrábida	S	[2]
				Sycon sp. Risso, 1827	Largo Rio Mira	S	[7]
Calcinea	Clathrinida	Clathrinidae Minchin, 1900	*Borojevia* Klautau, Azevedo, Cóndor-Luján, Rapp, Collins & Russo, 2013	*Borojevia cerebrum* (Haeckel, 1872)	Arrifana	S	[3]
			Clathrina Gray, 1867	*Clathrina blanca* (Miklucho-Maclay, 1868)	Apúlia	S	[5]
				Clathrina clathrus (Schmidt, 1864)	Algarve	S	[1]
					Arrifana	S	*[3]*
					Sagres	S	*[6]*
				Clathrina coriacea (Montagu, 1814)	Memória	I	*a*
					VN Mil Fontes	I	*a*
					Apúlia	S	[5]
					Arrábida	S	[2]
					Sines	S	[4]
				Clathrina blanca (Miklucho-Maclay, 1868)	Memória	I	*a*
					Prego	S	*a*
				Clathrina lacunosa (Johnston, 1842)	Arrifana	S	*[3]*
		Leucaltidae Dendy & Row, 1913	*Ascandra* Haeckel, 1872	*Ascandra contorta* (Bowerbank, 1866)	Arrábida	S	[2]
			Leucaltis Haeckel, 1872	*Leucaltis clathria* Haeckel, 1872	Apúlia	S	[5]
				Leucaltis nodusgordii (Poléjaeff, 1883)	Sines	S	[4]
				Leucetta solida (Schmidt, 1862)	Arrábida	S	[2]

Table 9-2. Bibliographic information on sponge diversity from the coast of Portugal – Class Demospongeae

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
Heteroscleromorpha	Axinellida	Axinellidae Carter, 1875	*Axinella* Schmidt, 1862	*Axinella* cf. *damicornis* (Esper, 1794)	Sagres	S	[7]
				Axinella damicornis (Esper, 1794)	Algarve	S	[1]
					Apúlia	S	[5]
					Arrábida	S	[8]
				Axinella guiteli Topsent, 1896	Algarve	S	[1]
					Sagres	S	[6]
				Axinella polypoides Schmidt, 1862	Algarve	S	[1]
					Arrábida	S	[2]
					Sagres	S	[7]
				Axinella verrucosa (Esper, 1794)	Arrábida	S	[2]
			Ophiraphidites Carter, 1876	*Ophiraphidites tortuosus* Carter, 1876	Cabo S. Vicente	S	[9]
			Phakellia Bowerbank, 1862	*Phakellia ventilabrum* (Linnaeus, 1767)	Cabo S. Vicente	S	[9]
					Porto	S	[10]
		Raspailiidae Nardo, 1833	*Eurypon* Gray, 1867	*Eurypon cinctum* Sarà, 1960	Arrábida	S	[8]
				Eurypon clavatum (Bowerbank, 1866)	Buarcos	I	[11]
					Magoito	I	[11]
				Eurypon coronula (Bowerbank, 1874)	Afife	I	[11]
			Janulum de Laubenfels, 1936	*Janulum spinispiculum* (Carter, 1876)	Cabo S. Vicente	S	[9]
			Raspailia Nardo, 1833	*R. (Clathriodendron) hispida* (Montagu, 1814)	Algarve	S	[1]
				Raspailia (Parasyringella) agnata (Topsent, 1896)	Apúlia	S	[5]
				Raspailia (Raspailia) ramosa (Montagu, 1814)	Arrábida	S	[8]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
				Raspailia (Raspailia) viminalis Schmidt, 1862	Arrábida	S	[2]
				Raspailia formidabilis Hanitsch, 1895	Sines	S	[4]
				Raspailia sp. Nardo, 1833	Cabo S. Vicente	S	[7]
		Stelligeridae Lendenfeld, 1898	*Paratimea* Hallmann, 1917	*Paratimea constellata* (Topsent, 1893)	Apúlia	S	[5]
			Stelligera Gray, 1867	*Stelligera rigida* (Montagu, 1814)	Amado	I	[11]
					Buarcos	I	[11]
					Galapos	I	[11]
					Magoito	I	[11]
					Memória	I	*a*
	Biemnida	Rhabderemiidae Topsent, 1928	*Rhabderemia* Topsent, 1890	*Rhabderemia intexta* (Carter, 1876)	Cabo S. Vicente	S	[9]
	Bubarida	Dictyonellidae van Soest, Diaz & Pomponi, 1990	*Acanthella* Schmidt, 1862	*Acanthella acuta* Schmidt, 1862	Arrábida	S	[8]
			Dictyonella Schmidt, 1868	*Dictyonella incisa* (Schmidt, 1880)	Algarve	S	[1]
					Arrábida	S	[8]
					Arrifana	S	[3]
				Dictyonella marsilii (Topsent, 1893)	Algarve	S	[1]
					Sagres	S	[6]
				Dictyonella obtusa (Schmidt, 1862)	Algarve	S	[1]
				Dictyonella pelligera (Schmidt, 1864)	Arrábida	S	[2]
			Tethyspira Topsent, 1890	*Tethyspira spinosa* (Bowerbank, 1874)	Afife	I	[11]
					Aguda	I	[11]
					Amado	I	[11]
					Buarcos	I	[11]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
					Galapos	I	[11]
					Ingrina	I	[11]
					Magoito	I	[11]
					Olhos d'Água	I	[11]
					Porto Côvo	I	[11]
	Clionaida	Clionaidae d'Orbigny, 1851	*Cliona* Grant, 1826	*Cliona celata* Grant, 1821	Afife	I	[11]
					Aguda	I	[11]
					Amado	I	[11]
					Buarcos	I	[11] a
					Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Magoito	I	[11]
					Memória	I	*a*
					Olhos d'Água	I	[11]
					Prego	S	*a*
					Apúlia	S	[5]
					Arrábida	S	[2, 8]
					Arrifana	S	[3]
					Largo Rio Mira	S	[7]
					Pelo Negro	S	*a*
					Sagres	S	[6]
					Viana Castelo	S	*a*

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
				Cliona lobata Hancock, 1849	Porto	S	[10]
				Cliona viridis (Schmidt, 1862)	Afife	I	[11]
					Aguda	I	[11]
					Amado	I	[11]
					Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Olhos d'Água	I	[11]
					Porto Côvo	I	[11]
					Algarve	S	[1]
					Arrábida	S	[2, 8]
					Arrifana	S	[3]
					Sagres	S	[6]
			Pione Gray, 1867	*Pione vastifica* (Hancock, 1849)	Afife	I	[11]
					Amado	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Olhos d'Água	I	[11]
					Arrábida	S	[2, 8]
		Placospongiidae Gray, 1867	*Placospongia* Gray, 1867	*Placospongia decorticans* (Hanitsch, 1895)	Apúlia	S	[5]
					Sines	S	[4]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
		Spirastrellidae Ridley & Dendy, 1886	*Diplastrella* Topsent, 1918	*Diplastrella bistellata* (Schmidt, 1862)	Arrábida	S	[8]
			Spirastrella Schmidt, 1868	*Spirastrella cunctatrix* Schmidt, 1868	Sagres	S	[6]
	Desmacellida	Desmacellidae Ridley & Dendy, 1886	*Desmacella* Schmidt, 1870	*Desmacella inornata* (Bowerbank, 1866)	Apúlia	S	[5]
	Haplosclerida	Callyspongiidae de Laubenfels, 1936	*Callyspongia* Duchassaing & Michelotti, 1864	*Callyspongia cylindrica* (Lendenfeld, 1886)	Cabo S. Vicente	S	[7]
					Leça	S	[4]
		Chalinidae Gray, 1867	*Chalinula* Schmidt, 1868	*Chalinula limbata* (Montagu, 1814)	Apúlia	S	[5]
				Chalinula renieroides Schmidt, 1868	Apúlia	S	[5]
			Haliclona Grant, 1841	*Haliclona (Gellius) angulata* (Bowerbank, 1866)	Arrábida	S	[8]
				Haliclona (Gellius) fibulata (Schmidt, 1862)	Apúlia	S	[5]
					Arrábida	S	[2]
					Sines	S	[4]
				H. (Halichoclona) fistulosa (Bowerbank, 1866)	Apúlia	S	[5]
				Haliclona (Haliclona) oculata (Linnaeus, 1759)	Apúlia	S	[5]
					Arrábida	S	[2]
				Haliclona (Haliclona) simulans (Johnston, 1842)	Aguda	I	*a*
					Buarcos	I	*a*
					Memória	I	*a*
					Viana Castelo	I	*a*
					Apúlia	S	[5]
					Arrábida	S	[8]
				Haliclona (Haliclona) sp. Grant, 1836	Buarcos	S	[4]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
					Sines	S	[4]
				Haliclona (Reniera) cinerea (Grant, 1826)	Apúlia	S	[5]
					Arrábida	S	[8]
					Sines	S	[4]
				H. (Reniera) mediterranea Griessinger, 1971	Arrábida	S	[8]
				Haliclona (Reniera) sp. Schmidt, 1862	Sines	S	[4]
				H. (Rhizoniera) indistincta (Bowerbank, 1866)	Apúlia	S	[5]
					Arrábida	S	[8]
				Haliclona (Rhizoniera) rosea (Bowerbank, 1866)	Buarcos	I	*a*
					Cabo S. Vicente	S	[7]
					Apúlia	S	[5]
				Haliclona (Rhizoniera) viscosa (Topsent, 1888)	Apúlia	S	[5]
					Arrábida	S	[8]
				H. (Soestella) valliculata (Griessinger, 1971)	Arrábida	S	[8]
				Haliclona (Soestella) xena De Weerdt, 1986	Apúlia	S	[5]
				Haliclona sp. Grant, 1841	Memória	I	*a*
					Prego	S	*a*
		Niphatidae van Soest, 1980	*Gelliodes* Ridley, 1884	*Gelliodes luridus* (Lundbeck, 1902)	Arrábida	S	[2]
		Petrosiidae van Soest, 1980	*Petrosia* Vosmaer, 1885	*Petrosia (Petrosia) ficiformis* (Poiret, 1789)	Arrábida	S	[2]
					Cabo S. Vicente	S	[7]
	Poecilosclerida	Acarnidae Dendy, 1922	*Acarnus* Gray, 1867	*Acarnus tortilis* Topsent, 1892	Arrábida	S	[8]
			Iophon Gray, 1867	*Iophon hyndmani* (Bowerbank, 1858)	Arrábida	S	[8]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
		Chondropsidae Carter, 1886	*Batzella* Topsent, 1893	*Batzella inops* (Topsent, 1891)	Arrábida	S	[8]
			Psammoclema Marshall, 1880	Psammoclema finmarchicum (Hentschel, 1929)	Apúlia	S	[5]
		Coelosphaeridae Dendy, 1922	*Coelosphaera* Thomson, 1873	*C. (Coelosphaera) phlyctenodes* (Carter, 1876)	Cabo S. Vicente	S	[9]
			Forcepia Carter, 1874	*Forcepia (Forcepia) forcipis* (Bowerbank, 1866)	Cabo S. Vicente	S	[9]
			Lissodendoryx Topsent, 1892	*L. (Lissodendoryx) isodictyalis* (Carter, 1882)	Amado	I	[11]
					Consolação	I	[11]
					Porto Côvo	I	[11]
					Arrábida	S	[2]
					Apúlia	S	[5]
		Crambeidae Lévi, 1963	*Crambe* Vosmaer, 1880	*Crambe crambe* (Schmidt, 1862)	Arrábida	S	[8]
		Crellidae Dendy, 1922	*Crella* Gray, 1867	*Crella (Crella) elegans* (Schmidt, 1862)	Algarve	S	[1]
					Arrábida	S	[2]
				Crella (Pytheas) donsi Burton, 1931	Apúlia	S	[5]
				Crella (Pytheas) fusifera Sarà, 1969	Algarve	S	[1]
					Arrábida	S	[8]
					Sagres	S	[6]
				Crella (Yvesia) albula (Bowerbank, 1866)	Apúlia	S	[5]
				Crella (Yvesia) pertusa (Topsent, 1890)	Amado	I	[11]
				Crella (Yvesia) rosea (Topsent, 1892)	Memória	I	*a*
			Crellomima Rezvoi, 1925	*Crellomima derma* Hentschel, 1929	Apúlia	S	[5]
		Esperiopsidae Hentschel, 1923	*Amphilectus* Vosmaer, 1880	*Amphilectus fucorum* (Esper, 1794)	Aguda	I	*a*
					Buarcos	I	*a*

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
					Esposende	I	*a*
					Memória	I	*a*
					Afife	I	[11]
					Amado	I	[11]
					Buarcos	I	[11]
					Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Magoito	I	[11]
					Apúlia	S	[5]
					Arrábida	S	[8]
			Ulosa de Laubenfels, 1936	*Ulosa stuposa* (Esper, 1794)	Arrábida	S	[2, 8]
		Hymedesmiidae Topsent, 1928	*Hemimycale* Burton, 1934	*Hemimycale columella* (Bowerbank, 1874)	Algarve	S	[1]
					Arrifana	S	[3]
					Sagres	S	[6]
			Hymedesmia Bowerbank, 1864	*H. (Hymedesmia) baculifera* (Topsent, 1901)	Ingrina	I	[11]
					Algarve	S	[1]
					Arrifana	S	[3]
				Hymedesmia (Hymedesmia) jecusculum	Memória	I	*a*
				Hymedesmia (Hymedesmia) pansa Bowerbank, 1882	Afife	I	[11]
					Consolação	I	[11]
					Arrábida	S	[8]
				H. (Hymedesmia) peachii Bowerbank, 1882	Arrábida	S	[8]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
				H. (Hymedesmia) pilata Bowerbank, 1882	Apúlia	S	[5]
				H. (Hymedesmia) versicolor (Topsent, 1893)	Arrábida	S	[8]
				Hymedesmia (Stylopus) coriacea (Fristedt, 1885)	Afife	I	[11]
					Buarcos	I	[11]
					Amado	I	[11]
					Algarve	S	[1]
				Hymedesmia (Stylopus) primitiva Lundbeck, 1910	Apúlia	S	[5]
				Hymedesmia (Stylopus) sp. Fristedt, 1885	Arrábida	S	[8]
			Phorbas Duchassaing & Michelotti, 1864	*Phorbas dives* (Topsent, 1891)	Afife	I	[11]
					Amado	I	[11]
					Consolação	I	[11]
					Galapos	I	[11]
					Porto Côvo	I	[11]
					Prego	S	*a*
					Arrábida	S	[8]
				Phorbas fictitius (Bowerbank, 1866)	Afife	I	[11]
					Amado	I	[11]
					Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Olhos d'Água	I	[11]
					Porto Côvo	I	[11]
					Ribeira das Ilhas	I	[12]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
					Algarve	S	[1, 13]
					Arrábida	S	[2, 8]
					Arrifana	S	[3]
					Sagres	S	[6]
				Phorbas plumosus (Montagu, 1814)	Afife	I	[11]
					Aguda	I	*a*
					Amado	I	[11]
					Buarcos	I	*a*
					Buarcos	I	[11]
					Consolação	I	[11]
					Ingrina	I	[11]
					Memória	I	*a*
					Olhos d'Água	I	[11]
					Apúlia	S	[5]
					Arrábida	S	[8]
				Phorbas tenacior (Topsent, 1925)	Algarve	S	[1]
					Arrifana	S	[3]
					Sagres	S	[6]
			Plocamionida Topsent, 1927	*Plocamionida ambigua* (Bowerbank, 1866)	Apúlia	S	[5]
				Plocamionida microcionides (Carter, 1876)	Cabo S. Vicente	S	[9]
		Microcionidae Carter, 1875	*Antho* Gray, 1867	*Antho (Antho) granditoxa* Picton & Goodwin, 2007	Memória	I	*a*
					Prego	S	*a*
				Antho (Antho) inconstans (Topsent, 1925)	Arrábida	S	[8]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
				Antho (Antho) involvens (Schmidt, 1864)	Afife	I	[11]
					Amado	I	[11]
					Ingrina	I	[11]
			Clathria Schmidt, 1862	*Clathria (Clathria) coralloides* (Scopoli, 1772)	Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Magoito	I	[11]
					Memória	I	*a*
					Viana Castelo	I	*a*
				Clathria (Clathria) toxistricta Topsent, 1925	Ribeira das Ilhas	I	[12]
				C. (Microciona) atrasanguinea (Bowerbank, 1862)	Cabo do Mundo	I	[14]
					Afife	I	[11]
					Aguda	I	[11]
					Buarcos	I	[11]
					Apúlia	S	[5]
				Clathria (Microciona) gradalis Topsent, 1925	Arrábida	S	[8]
				Clathria (Microciona) normani (Burton, 1930)	Apúlia	S	[5]
				C. (Microciona) spinarcus (Carter & Hope, 1889)	Arrábida	S	[8]
				Clathria (Microciona) strepsitoxa (Hope, 1889)	Aguda	I	[11]
					Buarcos	I	[11]
					Consolação	I	[11]
					Arrábida	S	[8]
				Clathria (Microciona) toxitenuis Topsent, 1925	Arrábida	S	[8]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
				Clathria (Paresperia) anchorata (Carter, 1874)	Apúlia	S	[5]
					Cabo S. Vicente	S	[9]
			Echinoclathria Carter, 1885	*Echinoclathria* sp. Carter, 1885	Sines	S	[4]
			Ophlitaspongia Bowerbank, 1866	*Ophlitaspongia papilla* Bowerbank, 1866	Aljezur	I	*a*
					Cabo do Mundo	I	[14]
					Memória	I	*a*
					Viana Castelo	I	*a*
		Mycalidae Lundbeck, 1905	*Mycale* Gray, 1867	*M. (Aegogropila) contarenii* (Lieberkühn, 1859)	Galapos	I	[11]
					Ingrina	I	[11]
					Olhos d'Água	I	[11]
					Porto Côvo	I	[11]
				Mycale (Aegogropila) rotalis (Bowerbank, 1874)	Apúlia	S	[5]
					Arrábida	S	[2]
				Mycale (Carmia) macilenta (Bowerbank, 1866)	Afife	I	[11]
					Arrábida	S	[8]
				Mycale (Carmia) minima (Waller, 1880)	Afife	I	[11]
					Consolação	I	[11]
				Mycale (Mycale) lingua (Bowerbank, 1866)	Algarve	S	[1]
				Mycale (Mycale) massa (Schmidt, 1862)	Cabo S. Vicente	S	[9]
		Myxillidae Dendy, 1922	*Myxilla* Schmidt, 1862	*Myxilla (Myxilla)* cf. *incrustans* (Johnston, 1842)	Arrábida	S	[2]
				M. (Myxilla) incrustans var. *viscosa* (Topsent, 1892)	Sines	S	[4]
				Myxilla (Myxilla) iotrochotina (Topsent, 1892)	Arrábida	S	[2]
				M. (Myxilla) macrosigma Boury-Esnault, 1971	Arrábida	S	[8]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
				Myxilla (Myxilla) rosacea (Lieberkühn, 1859)	Afife	I	[11]
					Amado	I	[11]
					Buarcos	I	[11]
					Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Magoito	I	[11]
					Memória	I	*a*
					Olhos d'Água	I	[11]
					Porto Côvo	I	[11]
					Prego	S	*a*
					Arrábida	S	[2, 8]
					Pelo Negro	S	*a*
		Tedaniidae Ridley & Dendy, 1886	*Tedania* Gray, 1867	*Tedania (Tedania) anhelans* (Vio in Olivi, 1792)	Amado	I	[11]
					Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Olhos d'Água	I	[11]
					Porto Côvo	I	[11]
					Arrábida	S	[2, 8]
				Tedania (Tedania) pilarriosae Cristobo, 2002	Memória	I	*a*
					Prego	S	*a*
					Viana Castelo	S	*a*

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
				Tedania (Tedania) suctoria (Schmidt, 1870)	Apúlia	S	[5]
			Trachytedania Ridley, 1881	*Trachytedania ferrolensis* Cristobo & Urgorri, 2001	Arrábida	S	[8]
	Polymastiida	Polymastiidae Gray, 1867	*Polymastia* Bowerbank, 1862	*Polymastia agglutinans* Ridley & Dendy, 1886	Memória	I	*a*
					Apúlia	S	[5]
				Polymastia boletiformis (Lamarck, 1815)	Memória	I	*a*
					Apúlia	S	[5]
				Polymastia mamillaris (Müller, 1806)	Afife	I	[11]
					Amado	I	[11]
					Buarcos	I	[11]
					Galapos	I	[11]
					Magoito	I	[11]
					Arrábida	S	[2]
				Polymastia penicillus (Montagu, 1814)	Memória	I	*a*
				Polymastia sp. Bowerbank, 1862	Memória	I	*a*
				Polymastia spinula Bowerbank, 1866	Apúlia	S	[5]
	Scopalinida	Scopalinidae Morrow, Picton, Erpenbeck, Boury-Esnault, Maggs & Allcock, 2012	*Scopalinidae* Schmidt, 1862	*Scopalina lophyropoda* Schmidt, 1862	Algarve	S	[1]
					Arrifana	S	[3]
					Sagres	S	[6]
	Suberitida	Halichondriidae Gray, 1867	*Axinyssa* Lendenfeld, 1897	*Axinyssa digitata* (Cabioch, 1968)	Algarve	S	[1]
			Ciocalypta Bowerbank, 1862	*Ciocalypta penicillus* Bowerbank, 1862	Algarve	S	[1]
					Arrábida	S	[8]
			Halichondria Fleming, 1828	*H. (Halichondria) bowerbanki* Burton, 1930	Apúlia	S	[5]
					Arrábida	S	[8]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
				Halichondria (Halichondria) genitrix (Schmidt, 1870)	Apúlia	S	[5]
				Halichondria (Halichondria) panicea (Pallas, 1766)	Afife	I	[11]
					Aguda	I	[11] a
					Amado	I	[11]
					Assafora	I	[12]
					Buarcos	I	[11] a
					Consolação	I	[11]
					Esposende	I	*a*
					Galapos	I	[11]
					Ingrina	I	[11]
					Magoito	I	[11]
					Memória	I	*a*
					Olhos d'Água	I	[11]
					Parede	I	[12]
					Porto Côvo	I	[11]
					Ribeira das Ilhas	I	[12]
					S Joao Estoril	I	*a*
					S. Bernardino	I	[12]
					Viana Castelo	I	*a*
					Apúlia	S	[5]
					Arrábida	S	[8]
				Halichondria sp. Fleming, 1828	Peniche	I	[15]
					Sines	S	[4]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
			Hymeniacidon Bowerbank, 1858	*Hymeniacidon perlevis* (Montagu, 1814)	Afife	I	[11]
					Aguda	I	[11] *a*
					Almograve	I	*a*
					Amado	I	[11]
					Angeiras	I	[14] *a*
					Apúlia	I	*a*
					Assafora	I	[12]
					Baleal	I	[12]
					Buarcos	I	[11] *a*
					Cabo do Mundo	I	[14]
					Consolação	I	[11]
					Esposende	I	*a*
					Galapos	I	[11]
					Ingrina	I	[11]
					Magoito	I	[11, 12]
					Memória	I	*a*
					Olhos d'Água	I	[11]
					Parede	I	[12]
					Peniche	I	[15]
					Porto Côvo	I	[11] *a*
					Ribeira das Ilhas	I	[12]
					S Joao Estoril	I	*a*
					Viana do Castelo	I	[12] *a*

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
					VN Mil Fontes	I	*a*
					Prego	S	*a*
					Apúlia	S	[5]
					Algarve	S	[1]
					Arrábida	S	[8], [2]
					S. Martinho Porto	S	[11]
					Sagres	S	[6]
					Sines	S	[4]
			Spongosorites Topsent, 1896	*Spongosorites difficilis* (Lundbeck, 1902)	Apúlia	S	[5]
			Vosmaeria Fristedt, 1885	*Vosmaeria crustacea* Fristedt, 1885	Apúlia	S	[5]
				Vosmaeria levigata Topsent, 1896	Apúlia	S	[5]
		Suberitidae Schmidt, 1870	*Aaptos* Gray, 1867	*Aaptos aaptos* (Schmidt, 1864)	Memória	I	*a*
				Aaptos papillata (Keller, 1880)	Afife	I	[11]
					Buarcos	I	[11]
					Memória	I	*a*
			Homaxinella Topsent, 1916	*Homaxinella subdola* (Bowerbank, 1866)	Apúlia	S	[5]
			Protosuberites Swartschewsky, 1905	*Protosuberites ectyoninus* (Topsent, 1900)	Arrábida	S	[8]
				Protosuberites epiphytum (Lamarck, 1815)	Afife	I	[11]
					Aguda	I	[11]
					Amado	I	[11]
					Buarcos	I	[11]
					Galapos	I	[11]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
					Olhos d'Água	I	[11]
					Apúlia	S	[5]
					Arrábida	S	[8]
				Protosuberites rugosus (Topsent, 1893)	Arrábida	S	[8]
			Pseudosuberites Topsent, 1896	*Pseudosuberites hyalinus* (Ridley & Dendy, 1886)	Apúlia	S	[5]
				Pseudosuberites mollis Topsent, 1925	Buarcos	I	[11]
					Algarve	S	[1]
					Apúlia	S	[5]
				Pseudosuberites sulphureus (Bowerbank, 1866)	Apúlia	S	[5]
			Suberites Nardo, 1833	*Suberites carnosus* (Johnston, 1842)	Afife	I	[11]
					Aguda	I	[11]
					Amado	I	[11]
					Buarcos	I	[11]
					Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Porto Côvo	I	[11]
					Arrábida	S	[8]
				Suberites massa Nardo, 1847	Apúlia	S	[5]
			Terpios Duchassaing & Michelotti, 1864	*Terpios fugax* Duchassaing & Michelotti, 1864	Amado	I	[11]
					Galapos	I	[11]
					Olhos d'Água	I	[11]
					Arrábida	S	[8]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
				Terpios sp. Duchassaing & Michelotti, 1864	Sagres	S	[6]
	Tethyida	Hemiasterellidae Lendenfeld, 1889	*Adreus* Gray, 1867	*Adreus fascicularis* (Bowerbank, 1866)	Afife	I	[11]
		Tethyidae Gray, 1848	*Tethya* Lamarck, 1815	*Tethya aurantium* (Pallas, 1766)	Amado	I	[11]
					Galapos	I	[11]
					Magoito	I	[11]
					Peniche	I	[15]
					Apúlia	S	[5]
					Arrábida	S	[2, 8]
					Lagos	S	[7]
					S. Martinho Porto	S	[11]
					Sagres	S	[6]
					Sines	S	[4]
		Timeidae Topsent, 1928	*Timea* Gray, 1867	*Timea mixta* (Topsent, 1896)	Afife	I	[11]
					Amado	I	[11]
					Buarcos	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Magoito	I	[11]
					Memória	I	*a*
					Olhos d'Água	I	[11]
				Timea unistellata (Topsent, 1892)	Arrábida	S	[8]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
	Tetractinellida	Ancorinidae Schmidt, 1870	*Dercitus* Gray, 1867	*Dercitus (Stoeba) plicatus* (Schmidt, 1868)	Arrábida	S	[2]
			Stelletta Schmidt, 1862	*Stelletta anancora* (Sollas, 1886)	Amado	I	[11]
					Consolação	I	[11]
					Galapos	I	[11]
					Porto Côvo	I	[11]
				Stelletta hispida (Buccich, 1886)	Amado	I	[11]
					Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Arrábida	S	[2, 8]
				Stelletta sp. Schmidt, 1862	Peniche	I	[15]
		Azoricidae Sollas, 1888	*Leiodermatium* Schmidt, 1870	*Leiodermatium pfeifferae* (Carter, 1873)	Cabo S. Vicente	S	[9]
		Calthropellidae Lendenfeld, 1907	*Calthropella* Sollas, 1888	*C. (Calthropella) geodioides* (Carter, 1876)	Cabo S. Vicente	S	[9]
		Geodiidae Gray, 1867	*Erylus* Gray, 1867	*Erylus discophorus* (Schmidt, 1862)	Amado	I	[11]
					Consolação	I	[11]
					Porto Côvo	I	[11]
					Arrábida	S	[2]
				Erylus mamillaris (Schmidt, 1862)	Amado	I	[11]
				Erylus mamillaris (Schmidt, 1862)	Ingrina	I	[11]
			Geodia Lamarck, 1815	*Geodia conchilega* Schmidt, 1862	Peniche	I	[15]
				Geodia cydonium (Linnaeus, 1767)	Amado	I	[11]
					Consolação	I	[11]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
					Porto Côvo	I	[11]
					Arrábida	S	[2]
				Geodia megastrella Carter, 1876	Cabo S. Vicente	S	[9]
				Geodia megastrella var. *laevispina* Carter, 1876	Cabo S. Vicente	S	[9]
			Pachymatisma Bowerbank in Johnston, 1842	*P. johnstonia* (Bowerbank in Johnston, 1842)	Apúlia	S	[5]
		Macandrewiidae Schrammen, 1924	*Macandrewia* Gray, 1859	*Macandrewia azorica* Gray, 1859	Cabo S. Vicente	S	[9]
		Pachastrellidae Carter, 1875	*Characella* Sollas, 1886	*Characella pachastrelloides* (Carter, 1876)	Cabo S. Vicente	S	[9]
				Characella tripodaria (Schmidt, 1868)	Sines	S	[4]
			Nethea Sollas, 1888	*Nethea amygdaloides* (Carter, 1876)	Cabo S. Vicente	S	[9]
			Triptolemma de Laubenfels, 1955	*Triptolemma intextum* (Carter, 1876)	Cabo S. Vicente	S	[9]
		Theonellidae Lendenfeld, 1903	*Discodermia* du Bocage, 1869	*Discodermia polydiscus* (Bowerbank, 1869)	Cabo S. Vicente	S	[9]
		Vulcanellidae Cárdenas, Xavier, Reveillaud, Schander & Rapp, 2011	*Poecillastra* Sollas, 1888	*Poecillastra compressa* (Bowerbank, 1866)	Apúlia	S	[5]
	Trachycladida	Trachycladidae Hallmann, 1917	*Trachycladus* Carter, 1879	*Trachycladus minax* (Topsent, 1888)	Afife	I	[11]
					Amado	I	[11]
					Magoito	I	[11]
					Arrábida	S	[8]
Keratosa	Dendroceratida	Darwinellidae Merejkowsky, 1879	*Aplysilla* Schulze, 1878	*Aplysilla rosea* (Barrois, 1876)	Afife	I	[11]
					Aguda	I	[11] a
					Amado	I	[11]
					Buarcos	I	[11]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
					Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Magoito	I	[12]
					Magoito	I	[11]
					Olhos d'Água	I	[11]
					Porto Côvo	I	[11]
				Aplysilla sp. Schulze, 1878	Cabo S. Vicente	S	[7]
					Sines	S	[4]
				Aplysilla sulfurea Schulze, 1878	Arrábida	S	[8]
		Dictyodendrillidae Bergquist, 1980	*Spongionella* Bowerbank, 1862	*Spongionella pulchella* (Sowerby, 1804)	Apúlia	S	[5]
					Algarve	S	[1]
					Arrábida	S	[8]
	Dictyoceratida	Dysideidae Gray, 1867	*Dysidea* Johnston, 1842	*Dysidea avara* (Schmidt, 1862)	Apúlia	S	[5]
					Algarve	S	[1]
					Lagos	S	[7]
					Sagres	S	[6]
				Dysidea fragilis (Montagu, 1814)	Afife	I	[11]
					Amado	I	[11]
					Consolação	I	[11]
					Memória	I	*a*
					Olhos d'Água	I	[11]
					Porto Côvo	I	[11]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
					Ribeira das Ilhas	I	[12]
					Prego	S	*a*
					Algarve	S	[1, 16]
					Apúlia	S	[5]
					Arrábida	S	[8]
					Arrifana	S	[3]
					Cabo Espichel	S	[16]
					Cabo S. Vicente	S	[7]
					Lagos	S	[7]
					Sagres	S	[7]
					Sagres	S	[6]
			Pleraplysilla Topsent, 1905	*Pleraplysilla spinifera* (Schulze, 1879)	Algarve	S	[1]
					Arrábida	S	[8]
					Cabo Espichel	S	[16]
					Lagos	S	[7]
		Irciniidae Gray, 1867	*Ircinia* Nardo, 1833	*Ircinia dendroides* (Schmidt, 1862)	Algarve	S	[1, 16]
					Arrábida	S	[16]
					Sagres	S	[6]
				Ircinia oros (Schmidt, 1864)	Algarve	S	[1]
					Arrábida	S	[8]
				Ircinia procumbens (Poléjaeff, 1884)	Mondego to Setúbal	S	[16]
				Ircinia sp. Nardo, 1833	Sagres	S	[6]
				Ircinia variabilis (Schmidt, 1862)	Memória	I	*a*

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
					VN Mil Fontes	I	*a*
					Arrábida	S	[2]
					Berlenga	S	[16]
					S. Martinho Porto	S	[16]
					Sines	S	[4]
			Sarcotragus Schmidt, 1862	*Sarcotragus fasciculatus* (Pallas, 1766)	Amado	I	[11]
					Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Olhos d'Água	I	[11]
					Porto Côvo	I	[11]
					Algarve	S	[1, 16]
					Apúlia	S	[5]
					Arrábida	S	[2, 8, 16]
					Arrifana	S	[3]
					Sagres	S	[6]
				Sarcotragus foetidus Schmidt, 1862	Algarve	S	[16]
				Sarcotragus spinosulus Schmidt, 1862	Amado	I	[11]
					Porto Côvo	I	[11]
					Algarve	S	[1, 16]
					Apúlia	S	[5]
					Arrábida	S	[8, 16]
					Sagres	S	[6]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
		Spongiidae Gray, 1867	*Coscinoderma* Carter, 1883	*Coscinoderma confragosum* Poléjaeff, 1884	Mondego to Setúbal	S	[16]
			Spongia Linnaeus, 1759	*Spongia (Spongia) agaricina* Pallas, 1766	Algarve	S	[1]
					Arrábida	S	[8, 12, 16]
					Arrifana	S	[3]
					Sagres	S	[6]
				Spongia (Spongia) irregularis (Lendenfeld, 1889)	Berlenga	S	[16]
					S. Martinho Porto	S	[16]
					Sines	S	[16]
				Spongia (Spongia) nitens (Schmidt, 1862)	Algarve	S	[16]
					Arrábida	S	[2, 16]
				Spongia (Spongia) officinalis Linnaeus, 1759	Amado	I	[11]
					Consolação	I	[11]
					Galapos	I	[11]
					Ingrina	I	[11]
					Olhos d'Água	I	[11]
					Porto Côvo	I	[11]
					Ribeira das Ilhas	I	[12]
					Apúlia	S	[5]
					Algarve	S	[1]
					Arrábida	S	[8, 16]
					Sagres	S	[6]
				S. (Spongia) osculata (Lendenfeld, 1889)	Sines	S	[4]
				Spongia (Spongia) virgultosa (Schmidt, 1868)	Algarve	S	[16]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
					Arrábida	S	[16]
				Spongia sp. Linnaeus, 1759	Lagos	S	[7]
		Thorectidae Bergquist, 1978	*Aplysinopsis* Lendenfeld, 1888	*Aplysinopsis* sp. Lendenfeld, 1888	Sines	S	[4]
			Cacospongia Schmidt, 1862	*Cacospongia mollior* Schmidt, 1862	Algarve	S	[16]
			Fasciospongia Burton, 1934	*Fasciospongia cavernosa* (Schmidt, 1862)	Algarve	S	[16]
			Hyrtios Duchassaing & Michelotti, 1864	*Hyrtios collectrix* (Schulze, 1880)	Sines	S	[4]
			Scalarispongia Cook & Bergquist, 2000	*Scalarispongia scalaris* (Schmidt, 1862)	Amado	I	[11]
					Consolação	I	[11]
					Olhos d'Água	I	[11]
					Porto Côvo	I	[11]
					Algarve	S	[16]
					Arrábida	S	[2, 8, 16]
					Lagos	S	[7]
Verongimorpha	Chondrillida	Chondrillidae Gray, 1872	*Thymosia* Topsent, 1895	*Thymosia guernei* Topsent, 1895	Aljezur	I	*a*
					Amado	I	[11]
					S. João Estoril	I	*a*
					Arrábida	S	[8]
		Halisarcidae Schmidt, 1862	*Halisarca* Johnston, 1842	*Halisarca dujardinii* Johnston, 1842	Apúlia	S	[5]
	Chondrosiida	Chondrosiidae Schulz, 1877	*Chondrosia* Nardo 1847	*Chondrosia reniformis* Nardo, 1847	Algarve	S	[1]
					Arrifana	S	[3]
					Sagres	S	[6]

Subclass	Order	Family	Genera	Species	Sampling Location	I/S	Reference
	Verongiida	Aplysinidae Carter, 1875	*Aplysina* Nardo, 1834	*Aplysina aerophoba* (Nardo, 1833)	Amado	I	[11]
					Consolação	I	[11]
					Ingrina	I	[11]
					Apúlia	S	[5]
					Arrábida	S	[8]
					Sagres	S	[6]
					Arrábida	S	[8, 16]
		Ianthellidae Hyatt, 1875	*Hexadella* Topsent, 1896	*Hexadella racovitzai* Topsent, 1896	Algarve	S	[1]

Table 9-3. Bibliographic information on sponge diversity from the coast of Portugal – Class Homoscleromorpha

Order	Family	Genera	Species	Sampling Location	I/S	Reference
Homosclerophorida	Oscarellidae Lendenfeld, 1887	*Oscarella* Vosmaer, 1884	*Oscarella cruenta* (Carter, 1876)	Cabo S. Vicente	S	[9]
			Oscarella lobularis (Schmidt, 1862)	Apúlia	S	[5]
				Algarve	S	[1]
				Arrábida	S	[8]
				Lagos	S	[7]
				Sagres	S	[6]
	Plakinidae Schulze, 1880	*Corticium* Schmidt, 1862	*Corticium candelabrum* Schmidt, 1862	Sagres	S	[6]
		Plakina Schulze, 1880	*Plakina monolopha* Schulze, 1880	Amado	I	[11]

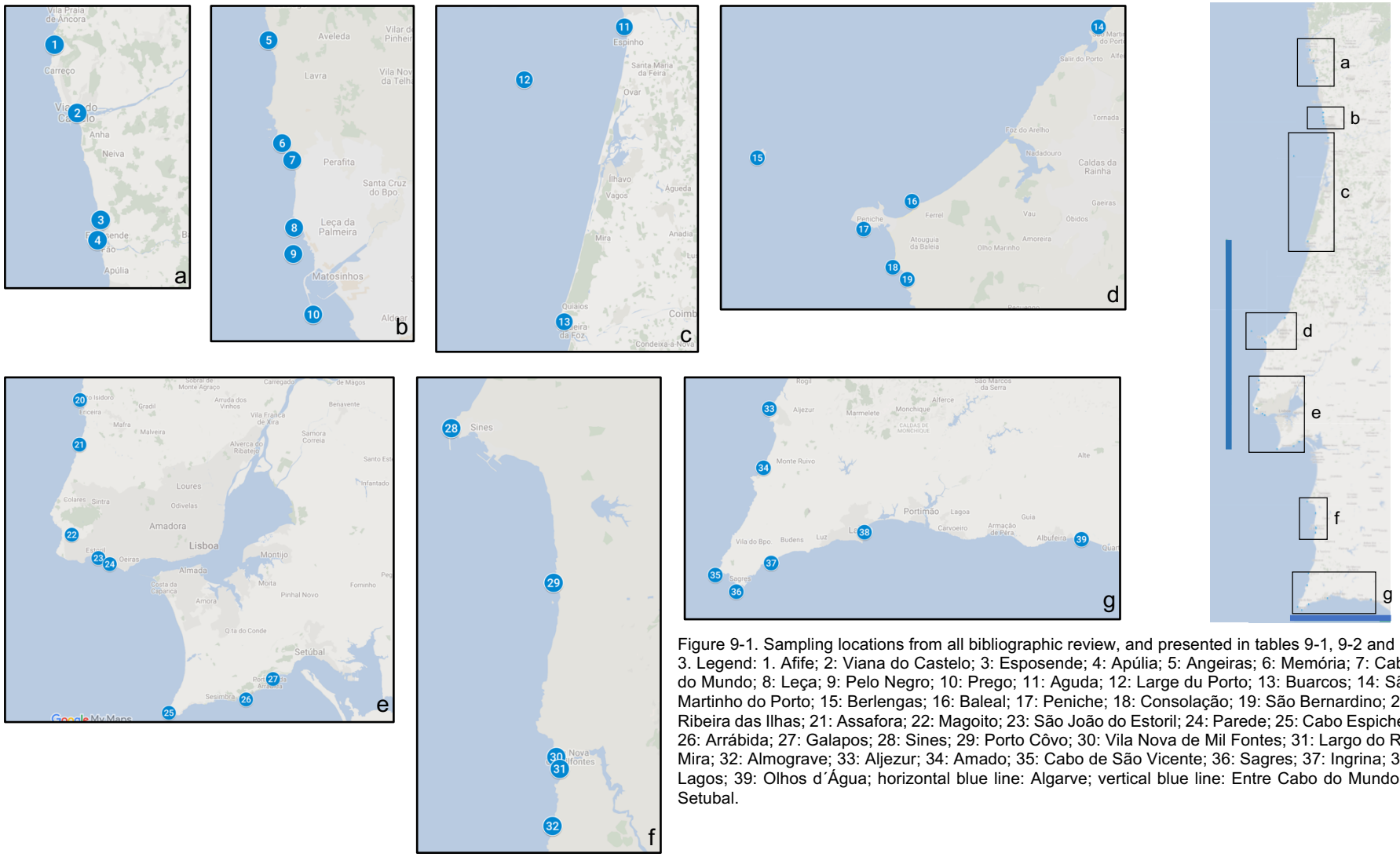

Figure 9-1. Sampling locations from all bibliographic review, and presented in tables 9-1, 9-2 and 9-3. Legend: 1. Afife; 2: Viana do Castelo; 3: Esposende; 4: Apúlia; 5: Angeiras; 6: Memória; 7: Cabo do Mundo; 8: Leça; 9: Pelo Negro; 10: Prego; 11: Aguda; 12: Large du Porto; 13: Buarcos; 14: São Martinho do Porto; 15: Berlengas; 16: Baleal; 17: Peniche; 18: Consolação; 19: São Bernardino; 20: Ribeira das Ilhas; 21: Assafora; 22: Magoito; 23: São João do Estoril; 24: Parede; 25: Cabo Espichel; 26: Arrábida; 27: Galapos; 28: Sines; 29: Porto Côvo; 30: Vila Nova de Mil Fontes; 31: Largo do Rio Mira; 32: Almograve; 33: Aljezur; 34: Amado; 35: Cabo de São Vicente; 36: Sagres; 37: Ingrina; 38: Lagos; 39: Olhos d´Água; horizontal blue line: Algarve; vertical blue line: Entre Cabo do Mundo e Setubal.

a *Personal data*

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Appendix II.

Book, brochure and poster for scientific divulgation of the most common sponges of the Portuguese intertidal area

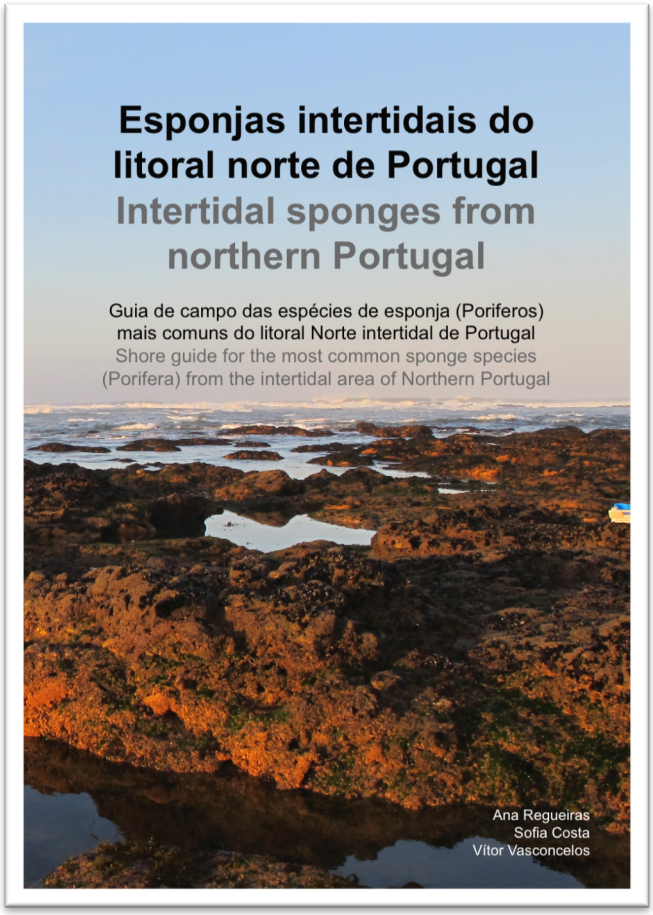
Book

Esponjas intertidais do litoral norte de Portugal

Guia de campo das espécies de esponja (Porifera) mais comuns do litoral Norte intertidal de Portugal

Intertidal sponges from the northern Portugal

Shore guide for the most common sponge species (Porifera) from the intertidal area of Northern Portugal



Esponjas intertidais do litoral norte de Portugal

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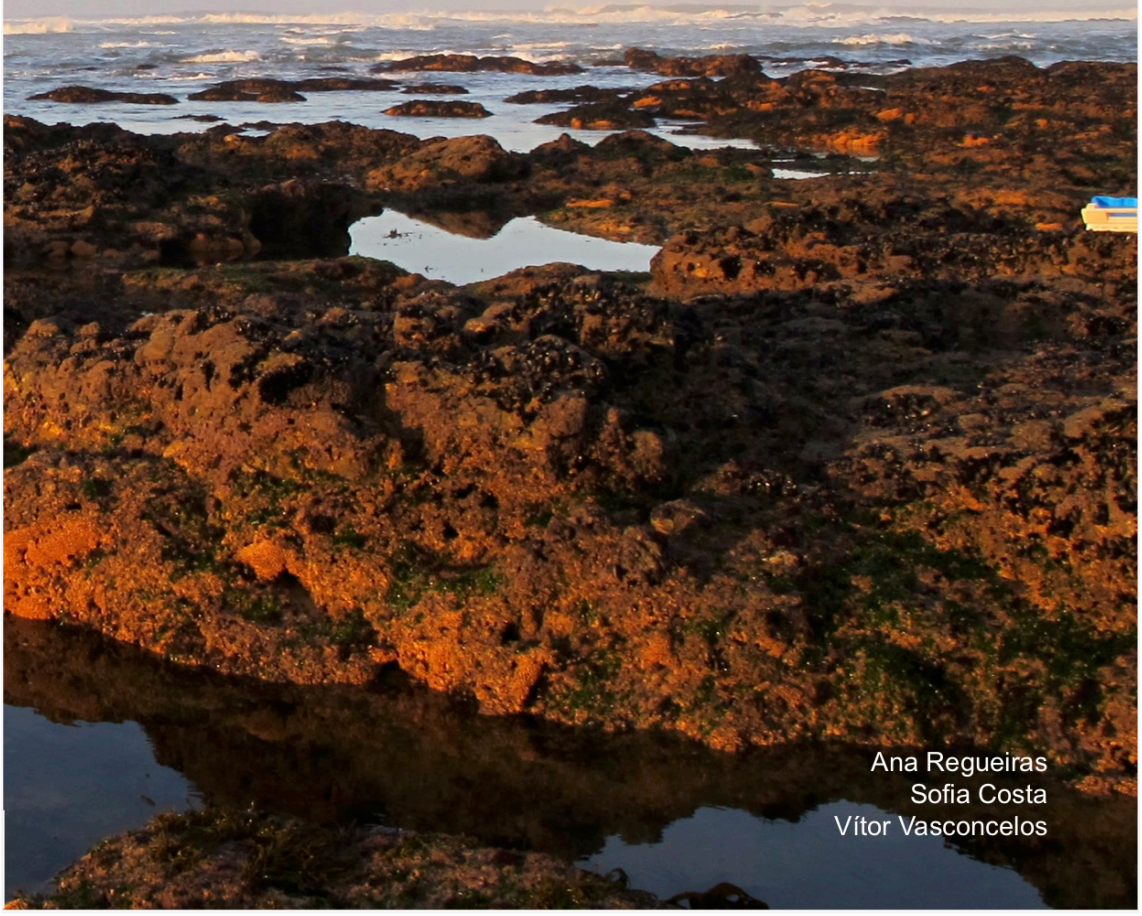

Ana Regueiras
Sofia Costa
Vítor Vasconcelos

INTRODUÇÃO

Portugal possui uma importante posição na costa Atlântica Europeia, onde o Mediterrâneo ainda exerce grande influência, resultando numa das mais interessantes regiões biogeográficas a nível europeu. O litoral norte de Portugal, caracteriza-se pela presença de um grande número de praias com afluências rochosas, substrato ideal para grande número de esponjas.

Este guia resulta de uma longa pesquisa das espécies de esponja intertidais presentes no litoral norte de Portugal. Contudo, apenas estão representadas algumas espécies, consideradas as mais comuns, de uma rica fauna de poríferos presentes nestas regiões.

INTRODUCTION

Portugal has a unique location on the European Atlantic coast, where the Mediterranean still has its influence, resulting in one of the most interesting European biogeographic regions. The northern Portuguese sea shore is characterized by the presence of various rocky beaches, the ideal substrate for sponge settlement.

This guide results from a long research of the intertidal sponges inhabiting the rocky sea shore of northern Portugal. Here are only represented some of them, the most common species of a rich fauna of Porifera that appears in these areas.

AS ESPONJAS

As esponjas são animais pertencentes ao Filo Porifera, que datam de há cerca de 600 milhões de anos, constituindo o ramo menos evoluído dos Metazoários. Estes animais contribuíram para a construção dos recifes. Estudos recentes apontam também para um possível papel das esponjas no aumento do oxigénio nos oceanos e, consequentemente, para a explosão de formas de vida mais complexas. São organismos aquáticos, sesseis, sem verdadeiros tecidos ou órgãos, onde as células conservam a totipotência, sendo que as funções vitais são asseguradas por células mais ou menos especializadas. São conhecidas cerca de 8000 espécies, amplamente distribuídas. São maioritariamente marinhas, embora também surjam em água doce.
O corpo é suportado por elementos esqueléticos de sílica ou carbonato de cálcio, as espículas, que podem estar ausentes, e por elementos orgânicos, principalmente fibras de espongina.

NUTRIÇÃO

As esponjas são animais filtradores, com a superfície perfurada por inúmeros poros inalantes, ostíolos e por poros exalantes, em menor número e de maiores dimensões, os ósculos. Algumas esponjas podem filtrar até 20000 vezes o seu volume de água por dia.
A circulação da água no interior da esponja deve-se à presença de células flageladas que se movimentam de forma sincronizada, os coanócitos. Muitas esponjas têm a capacidade de regular a quantidade de água que entra, por contração de células contrácteis, os porócitos. A contração ocorre normalmente como resposta a estímulos físicos e/ou à remoção do animal da água. Na presença de um intruso, podem igualmente sessar por completo a circulação da água, por paragem da movimentação dos coanócitos. O mecanismo pelo qual se dá a passagem desta informação é ainda desconhecido, uma vez que as esponjas são desprovidas de um sistema nervoso. Os coanócitos são igualmente responsáveis pela filtração da água e captação dos nutrientes, por fagocitose, e transferindo-as para outras células, os arqueócitos , que se encarregam da sua digestão. A presença de organismos

THE SPONGES

Sponges are animals, belonging to the Phylum Porifera. They appeared around 600 million years ago, and constitute the bottom (less evolved) of the Metazoan branch. They contributed to the formation of the reefs. Recent studies also point to their role in the increase of oxygen on the oceans, a requisite for the explosion of more complex life forms on Earth. They are sessile, aquatic organisms, without true tissues or organs, constituted by cells that maintain their totipotency, and that are more or less specialized to maintain vital functions. There are around 8000 different species described, worldwide distributed. The majority are marine but can also occur in freshwater. The body is supported by a silica or calcium carbonate skeleton, called spicules, that can be absent, and by organic elements, mainly spongin fibbers.

NUTRITION

Sponges are filter feeder animals. Surface is covered with numerous inhalant apertures, ostia and exhalant apertures, oscules. Normally, oscules are less and bigger than ostia. Some sponges can filter up to 20000 times their volume of water per day. The circulation of water through the sponge is due to the synchronized movement of flagellated cells, choanocytes. Some sponges have the ability to regulate the amount of water that enters, contracting some cells, known as porocytes. This contraction normally occurs in response to physical stimuli and/or removal from the water. When feeling threatened, sponges can also completely stop water circulation, through intermission of the movement of the choanocytes. It is still unknown the mechanism responsible for this signalling, since the sponges are devoid of a nervous system. Water filtration and nutrient captation is done by the choanocytes through phagocytosis, and then transfer to the archaeocytes, where digestion takes place.
The presence of symbiont organisms, such as algae, bacteria and cyanobacteria are equally important in the nutritional process of the sponge, providing the animal with important

em simbiose com a esponja, nomeadamente de algas, bactérias e cianobactérias são igualmente importantes no processo nutricional da esponja, fornecendo ao animal metabolitos importantes para a sua sobrevivência.

REPRODUÇÃO

A reprodução pode ser sexuada ou assexuada, estando intimamente relacionada com condições ambientais. A reprodução assexuada permite o desenvolvimento rápido de novos indivíduos similares à esponja parental, assim como a formação de gémulas, capazes de sobreviver em condições adversas e depois desenvolver-se quando as condições forem mais favoráveis. Na reprodução sexuada, surge variação genética, pois existe fecundação de gâmetas. Neste tipo de reprodução, há formação de larvas, de vida livre, o que permite a colonização de novas superfícies.

INTERESSE ECOLÓGICO E COMERCIAL

O conhecimento da diversidade de Poriferos tem uma enorme importância ecológica.
As esponjas são essencialmente conhecidas devido ao seu uso como esponjas de banho. Nos últimos anos, no Mar Mediterrâneo, tem havido uma sobre-exploração das espécies usadas para este fim (*Euspongia officinalis* e *Hippospongia communis*), pondo em risco a sobrevivência das mesmas.
Estes animais, filtradores ativos, formam relações de simbiose com outros organismos. Das associações com cianobactérias, bactérias e fungos, resulta na produção de compostos bioactivos com elevado interesse farmacêutico e/ou toxicológico. Alguns compostos extraídos das esponjas já são atualmente usados na indústria farmacêutica, como o Aciclovir (Ara A), para tratamento de herpes, a Citarabina (Ara C), usado no tratamento de algumas leucemias e linfomas, e o AZT, antirretroviral HIV. Outros compostos, como a Halichondrina B, possuem propriedades anticancerigenas, encontrando-se em diversas fases de diferentes ensaios clínicos e pré-clinicos. Contudo, problemas ecológicos surgem na pesquisa e extração destes compostos. Por exemplo, para obter 12,5 mg de Halichondrina B, são necessários cerca de 600 kg de esponja. Com vista a combater este problema de sobre-exploração de algumas espécies, nos últimos

metabolites.

REPRODUCTION

Sexual and asexual reproduction are intimately connected with environmental conditions. Asexual reproduction allows quick development of new organisms similar to the parental sponge. It also allows the production of gemmules, capable of surviving through adverse environmental conditions. Sexual reproduction consists in the fecundation of gametes, and therefore, genetic variation. In this type of reproduction, there is a free-living larvae stage, allowing the colonization of new habitats.

ECOLOGIC AND COMMERCIAL INTEREST

Understanding the diversity of Porifera has an enormous ecological importance.
The main use of marine sponges is as bath sponges. In the last few years, in the Mediterranean, it has been an over-exploration of the species used for this purpose (*Euspongia officinalis* and *Hippospongia communis*).
These animals are active filter feeders, forming symbiosis with other organisms. Associations with cyanobacteria, bacteria and fungus can lead to the production of bioactive compounds with pharmaceutical and/or toxicological interest. Some compounds extracted from marine sponges and already being used by the pharmaceutical industry are Acyclovir (Ara A), used for herpes treatment, Cytarabine (Ara C), used for leukaemia and lymphoma treatment, and AZT, a HIV anti-retroviral. Other compounds, like Halichondrin B, are known to have anticancer properties, being at the moment in several phases of clinical or preclinical trials. The extraction of these compounds arises some ecological problems. For example, to extract 12,5 mg of Halichondrin B, it is needed around 600 kg of sponge. In order to avoid over-exploration the some species, it has been developed in the last few years other ways to obtain the compound, mainly through sponge aquaculture and synthetic production of the compounds.

anos tem-se recorrido tanto à produção em aquacultura das esponjas, bem como tentar desenvolver formas de síntese sintética destes compostos.
Além do seu interesse como produtores de compostos bioativos, também podem ser usados como bioindicadores da qualidade da água. Estes animais estão diretamente dependentes da qualidade ambiental, devido ao facto de serem filtradores e sésseis. Logo, conhecendo a diversidade existente em determinado local, é possível inferir sobre a qualidade da água.
Uma vez que são os animais com uma estrutura mais simples, também podem ser excelentes modelos para diversos estudos.
A legislação portuguesa não contempla a proteção de nenhuma espécie de esponja. As únicas espécies protegidas referem-se às referidas na Convenção de Berna (Conservação da Vida Selvagem e do Meio Natural da Europa), no anexo II (espécies estritamente protegidas) e no anexo III (espécies protegidas), sendo todas espécies do Mediterrâneo.

IDENTIFICAÇÃO

Algumas esponjas possuem características marcantes, que permitem a sua identificação *in situ* mas, a maioria, exige que sejam recolhidas amostras e analisadas em laboratório. As espículas presentes nos organismos são essenciais para a identificação das espécies e, de acordo com o seu tamanho dividem-se em megascleras e microscleras. Outro fator importante na identificação tem a ver com a forma como as espículas e as fibras de espongina se dispõem para formar o esqueleto interno.

Na região intertidal, as esponjas encontram-se sob superfícies rochosas ou arenosas, com pelo menos uma parte do dia submersas e, normalmente, em zonas protegidas da luz solar direta. Características como cor, forma, consistência, presença de muco, cheiro e tipo de substrato são importantes na identificação destes organismos. Como algumas destas características alteram-se após a coleta, é recomendado que sejam documentadas *in situ.*
Uma vez que as esponjas podem produzir substâncias tóxicas ou possuir espículas projetadas para o exterior do corpo, é aconse-

Besides their importance as bioactive compounds producers, sponges can also be used as water quality bioindicators. Because these animals are filter feeders, they are completely dependent from the environment and, understanding sponge diversity allows to infer water quality.
Once they have a simple structure, Porifera can also be used as an animal model for scientific studies.
Portuguese legislation doesn't protect any sponge in particular. The only Porifera species protected in the European Union are the ones in the Berne Convention (Convention on the Conservation of European Wildlife and Natural Habitats), appendix II (strictly protected fauna species) and III (protected fauna species) and, all of them are Mediterranean species.

IDENTIFICATION

Some sponges have distinctive characters, allowing them to be identified *in situ*. But the majority needs to be analysed in a laboratory in order to identify them. Spicules are essential for sponge identification. According to their size, spicules can be separated into two categories: megascleres and microscleres. Other important character in sponge identification is the internal skeleton, the way spicules and spongin fibbers are arranged.

In the intertidal areas, sponges are in rocky or sandy surfaces, with at least a part of the day submerged and, normally, protected from direct sunlight. Characters like colour, shape, consistency, presence of mucous, smell and type of substrate are important for Porifera identification. Some of these characters can change after collection so, it is important to document all of them *in situ.* Sponges can produce toxic substances or have spicules projected from the surface, being important to always use gloves when handling them. When collecting these organisms, it is essential to properly accommodate them in plastic bags

lhado sempre o uso de luvas para o manuseio dos organismos. No caso de recolha de espécimes, estes devem ser acondicionados em sacos que vedem ou frascos, e submersos em água do mar.

Para estudos taxonómicos, os espécimes devem ser colocados em etanol a 90%, até 24h após a coleta. Para conservação a longo prazo, deve-se depois transferir-se os organismos para etanol a 70%.

TÉCNICAS DE IDENTIFICAÇÃO

A observação microscópica de espículas e do arranjo do esqueleto são parâmetros fundamentais para a identificação das espécies. Assim, seguem indicações para visualização microscópica rápida destas características:

Preparação rápida para visualização de espiculas por microscopia:
Numa lâmina colocar uma pequeno fragmento de esponja, cobri-lo com uma lamela e deitar umas gotas de solução de hipoclorito de sódio, deixando que a peça se dissolva. Lavar depois com água várias vezes, usando papel de filtro para absorver a água. Finalmente fazer uma lavagem com álcool, e deixar secar sobre uma placa aquecedora.

Cortes espessos rápidos para visualização do esqueleto :
É aconselhado que as esponjas sejam mantidas em álcool por pelo menos 24 horas antes de se proceder aos cortes. Fazer cortes relativamente finos (< 0,5mm) da esponja, tanto longitudinais como transversais. Os cortes devem ser feitos usando uma lâmina de bisturi bem afiada. Colocar os cortes imersos em álcool absoluto, num vidro de relógio por aproximadamente 15 minutos, para garantir que toda a água é removida dos tecidos. Posteriormente, colocá-los sob uma lâmina, distinguindo entre os cortes longitudinais e os transversais, e deixar secar. Se os cortes tiverem tendência a enrolar ao secar, re-hidratar com etanol e colocar lamelas e um pequeno peso sobre os cortes durante a secagem. Estes cortes podem ser imediatamente observados por microscopia, ou pode-se usar uma resina sintética para os preservar por longos períodos.

or flasks with natural sea water and transport them refrigerated.

For taxonomical studies, specimens should then be submerged in 90% ethanol, until 24h after collection. To preserve them for longer periods, it is best to then change the animal to 70% ethanol.

TECHNIQUES FOR IDENTIFICATION

As said before, spicules and skeleton microscopic observation are essential characters for species identification. Here are some directions for a quick microscopic visualization:

Quick preparation of spicules:
On a slide put a small sponge fragment, covering it with a coverslip. Put a few drops of sodium hypochlorite on the slide, letting the piece to dissolve. After, wash it a few times with water, using filter paper to absorb the water. At the end, make a final wash with ethanol and leave it to dry.

Quick cuts for skeleton observation:
Prior to make the cuts, leave the sponge in ethanol, for at least 24 hours. Make relatively thin cuts of the sponge (<0,5mm), both longitudinal and transverse. The cuts must be made using a very sharp scalpel or razor blade. In a watch-glass, submerge the cuts in 100% ethanol for approximately 15 minutes, to make sure all the water is removed from the tissues. After that, put the cuts on a slide, making sure to distinguish between longitudinal and transverse cuts, and let them dry. If the cuts start to curl, re-hydrate them with ethanol and then let them dry putting a coverslip and a small weight on top of the cuts. After drying, the cuts can be visualized under the microscope or, to preserve for longer periods, they can be mounted with a synthetic resin.

CLASSIFICAÇÃO

O Filo Porifera divide-se em três classes:

Calcária
Exclusivamente marinhas. Com esqueleto mineral inteiramente composto por carbonato de cálcio. As espículas são bi, tri ou tetra radiadas. Não possuem microscleras. Descritas cerca de 800 espécies.

Hexactinellida
Com esqueleto silicioso e espículas com 6 raios. Conhecidas como esponjas de vidro, ocorrem normalmente em águas profundas. Existem cerca de 600 espécies descritas.

Demospongiae
Esponjas compostas por um esqueleto de espículas siliciosas e/ou fibras de espongina. As espículas podem estar ausentes. Compreendem cerca de 85% de todas as espécies de Porifera descritas. A maioria são marinhas, ocorrendo a todas as profundidades. Existem também espécies de água doce.

Neste guia apenas estão presentes espécies das Classes Calcária e Demospongiae.

CLASSIFICATION

Phylum Porifera is divided into 3 classes:

Calcaria
Exclusively marine species. Mineral skeleton entirely of calcium carbonate. Skeletal elements are di, tri and tetractines. There are no microscleres. There are around 800 described species.

Hexactinellida
Silicious skeleton with 6 rayed spicules. Known as glass sponges and normally occur in deep waters. There are around 600 different species described.

Demospongiae
Silicious spicules and/or spongin fibbers. Spicules can be absent. They comprise about 85% of the Poriferan. Most marine occurring at all depths, but can also occur in freshwater habitats worldwide.

This guide only comprises species from Calcaria and Demospongiae classes.

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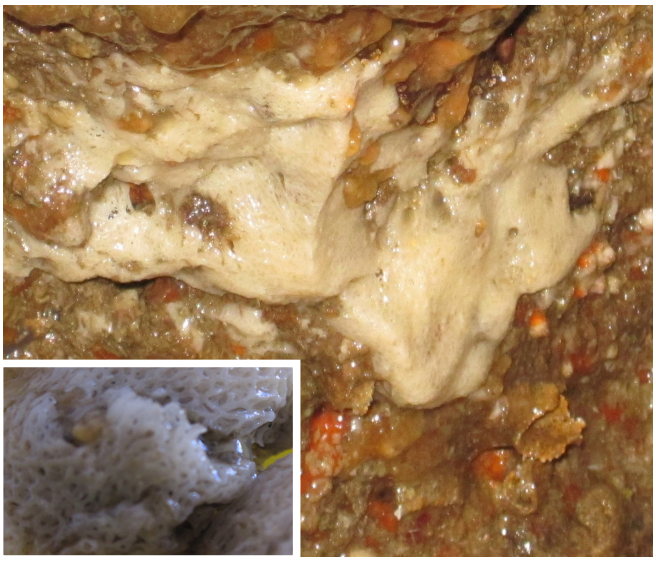
GUIA DE ESPONJAS
SPONGE GUIDE

Como usar este guia
How to use this guide



Clathrina coriacea
(Montagu, 1814)

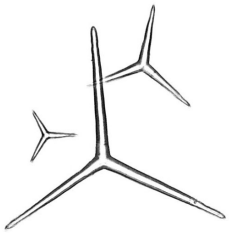
Classe Class
Calcarea
Subclasse Subclass
Calcinea
Ordem Order
Clathrinida
Família Family
Clathrinidae

DESCRIÇÃO
DESCRIPTION

Cor: Amarelo pálido ou branco amarelado (amarela em álcool).
Forma: Pequenas "almofadas". Constituída por estrutura tubular tridimensional compacta em forma de trelissa.
Consistência: Delicada, compressível, frágil.
Superfície: Suave. Estrutura tubular forma ósculos, ligeiramente elevados da superfície.
Habitat: Águas rasas, superfícies rochosas limpas, sob pedras ou em fendas.

Colour: White to pale yellow (becomes yellow in alcohol).
Shape: Small cushions. Formed by a tightly knit trelliswork of tubes.
Consistency: Soft, compressible, fragile, delicate.
Surface: Smooth. Tubular structure forms the oscules, slightly elevated from the surface.
Habitat: Common in shallow subtidal under overhangs and in the intertidal under boulders and in crevices.

ESPICULAS
SPICULES



Espículas calcárias do tipo triactina de ângulos iguais

Calcareous spicules with regular triacines, equally angled

Aaptos papillata
(Keller,1880)

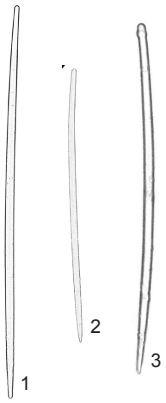
Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Suberitida
Família Family
Suberitidae

DESCRIÇÃO
DESCRIPTION

Cor: Tons de violeta e vermelho. Extremidade das pápilas mais claras. Internamente com tom alaranjado.
Forma: Hemisférica, ou em forma de almofada.
Consistência: Firme e difícil de retirar do substrato.
Superfície: Ligeiramente híspida com numerosas pápilas.
Habitat: Enterrada na areia. Apenas detetável pelas pápilas visíveis à superfície.

Colour: Shades of violet, lighter at papillae tips. Orange internally.
Shape: Hemispheric or pillow shape.
Consistency: Firm. Hard to remove from the substrate.
Surface: Slightly hispid with numerous papillae.
Habitat: Buried on the sand, detectable only by the papillae sticking out.

ESPICULAS
SPICULES



Megascleras:
1. Estrongilos
2. Estilos (pequenos)
3. Tilostilos (intermédios)
Microscleras:
Ausentes

Megascleres:
1. Strongyloxeas
2. Styles (small)
3. Tylostyles (medium size)
Microscleres:
Absent

Polymastia agglutinans

Ridley and Dendy, 1886

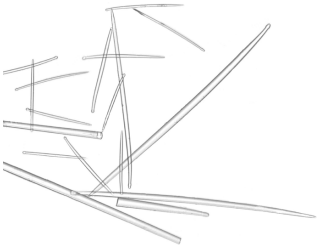
Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Polymastiida
Família Family
Polymastiidae

DESCRIÇÃO
DESCRIPTION

Cor: Amarelo a laranja.
Forma: Forma de almofada com pápilas à superfície.
Consistência: O corpo e as papilas são duros e firmes.
Superfície: Com papilas finas. Numerosas partículas (areia, restos de conchas, etc.) incrustadas à superfície.
Habitat: Sob camadas de sedimentos.

Colour: Yellow to Orange.
Shape: Cushion with papillae.
Consistency: Firm.
Surface: With thin papillae. Characterized by the presence of foreign material (shell debris, sand etc.) incrusted to the surface.
Habitat: Sandy bottoms.

ESPICULAS
SPICULES



Megascleras:
Tilóstilos em três tamanhos diferentes
Microscleras:
Ausentes

Megascleres:
Tylostyles in three different sizes
Microscleres:
Absent

Polymastia penicillus
(Montagu, 1814)

Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Polymastiida
Família Family
Polymastiidae

DESCRIÇÃO
DESCRIPTION

Cor: Corpo com cor cinzento escuro ou laranja amarelado. Papilas são amarelas pálidas.
Forma: Forma de almofada, com papilas que se projetam do corpo, enterrado no substrato.
Consistência: O corpo e as papilas são duros.
Superfície: Corpo híspido. Os poros e os ósculos encontram-se nas extremidades das papilas. As papilas exalantes são mais largas e em menor número.
Habitat: Sob camadas de sedimentos, ficando a superfície do corpo enterrada na areia, firmemente agarrado à rocha por baixo dos sedimentos.

Colour: Body is greyish or orange yellow. Papillae are pale yellow.
Shape: Cushion, with papillae projecting from the sediment covered body.
Consistency: Body hard and papillae stiff.
Surface: Body hispid. Oscules and pores on the papillae. The exhalant papillae are larger and fewer in number.
Habitat: Body beneath a layer of surface, firmly attached to the rocks beneath the sediments.

ESPICULAS
SPICULES

Megascleras:
Tilóstilos em três tamanhos diferentes
Microscleras:
Ausentes

Megascleres:
Tylostyles in three different sizes
Microscleres:
Absent

Cliona celata
Grant, 1826

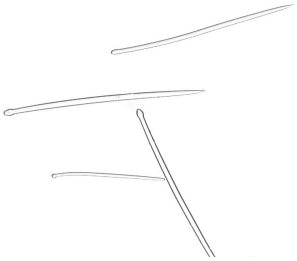
Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Clionaida
Família Family
Clionaidae

DESCRIÇÃO
DESCRIPTION

Cor: Amarela. Escurece fora de água. Em álcool pode ficar castanha.
Forma: Tem duas formas distintas: uma perfurante com papilas amarelas visíveis através das rochas calcárias; outra grande e massiva com papilas caracteristicamente achatadas.
Consistência: Compacta e firme.
Superfície: Macia. Possui uma camada externa mais dura, coberta por papilas inalantes retrácteis. Fora de água as papilas retraem-se e fecham. Com ósculos grandes.
Habitat: Rochas. O início de vida (forma perfurante) pode ser em pedras calcárias, conchas ou algas vermelhas.

Colour: Yellow, becoming darker outside of water and brown in alcohol.
Shape: With 2 distinct forms: one boring form with yellow papillae sticking out of limestone; other large massive, with characteristic flattened papillae.
Consistency: Firm and compact.
Surface: Smooth, with an outer layer tougher. Covered with inhalant retractable papilla. These papillae close and retract, becoming unnoticeable outside of the water. With big oscules.
Habitat: Massive form occurs on rock. Begins life by boring into limestone, shells or calcareous red algae.

ESPICULAS
SPICULES



Megascleras:
Tilóstilos com típica região inchada mesmo antes da extremidade
Microscleras:
Ausentes

Megascleres:
Tylostyles with swallen heads just bellow the tip
Microscleres:
Absent

Stelligera rigida
(Montagu, 1814)

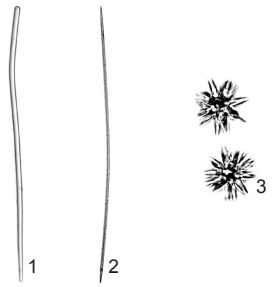
Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Axinellida
Família Family
Stelligeridae

DESCRIÇÃO
DESCRIPTION

Cor: Amarelo pálido a laranja.
Forma: Ramificada, com extremidades em forma de bolbos.
Consistência: Firme.
Superfície: Híspida. Ouriçada devido a longas espículas que se projetam à superfície. Ósculos pequenos.
Habitat: Locais abrigados mas com alguma corrente.

Colour: Pale yellow to orange.
Shape: Branching-erect, with bulbous-like extremities.
Consistency: Firm.
Surface: Strongly hispid. Bristly due to long projecting spicules. With small oscules.
Habitat: Sheltered locations with some current.

ESPICULAS
SPICULES



Megascleras:
1. Estilos de diferentes tamanhos
2. Oxeas de diferentes tamanhos
Microscleras:
3. Euásteres

Megascleres:
1. Styles in different sizes
2. Oxeas in different sizes
Microscleres:
3. Euasters

Clathria sp.

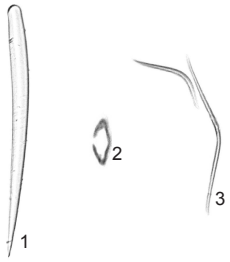
Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Poecilosclerida
Família Family
Microcionidae

DESCRIÇÃO
DESCRIPTION

Cor: Vermelho acastanhado.
Forma: Incrustante.
Consistência: Suave, facilmente quebrável.
Superfície: Microhíspida, aveludada.
Habitat: Rochas. Mais comum de águas profundas.

Colour: Red to brown.
Shape: Incrusting.
Consistency: Smooth, brittle.
Surface: Finely hispid, velvety.
Habitat: Rocky surfaces. Most common in deep waters.

ESPICULAS
SPICULES



Megascleras:
1. Subtilóstilos
Microscleras:
2. Isoquelas palmadas
3. Toxas de dois tamanhos distintos

Megascleres:
1. Subtilostyles
Microscleres:
2. Palmate Isochelae
3. Toxa in two distict sizes

Ophlitaspongia papilla

Bowerbank, 1866

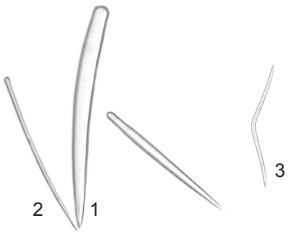
Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Poecilosclerida
Família Family
Microcionidae

DESCRIÇÃO
DESCRIPTION

Cor: Laranja a vermelho forte. Quando espremida, liberta o pigmento.
Forma: Finos tapetes ou com forma de almofada.
Consistência: Forte e elástica. Compressível. Parte-se com uma bolacha pouco dura.
Superfície: Homogénea, ligeiramente granulada, híspida. Com numerosos ósculos distribuídos por toda a superfície.
Habitat: Rochas, conchas. Associada a algas como *Fucus* e *Laminaria*. Encontra-se em áreas com forte movimento de água.

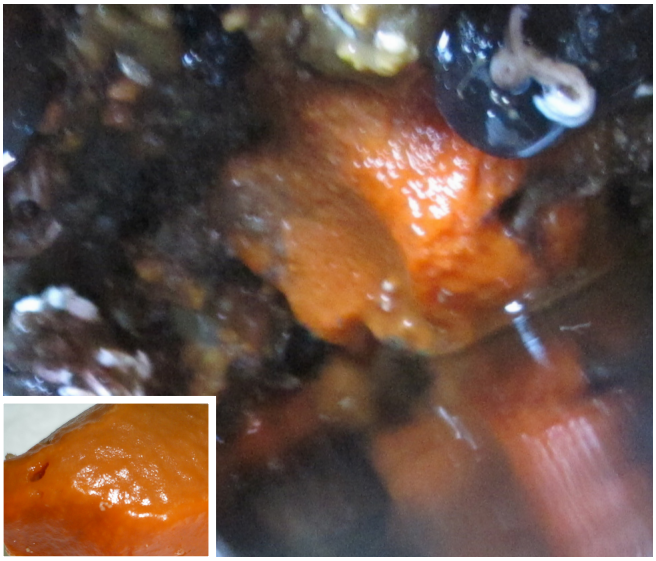
Colour: Bright Orange-red. The pigment is released when squeezed.
Shape: Thin sheets. Can develop into cushions.
Consistency: Firm and elastic. Compressible. Brakes like a soft cookie.
Surface: Even, very finely granular, hispid. With numerous oscules evenly distributed.
Habitat: On rock or shells. Commonly associated with the algae Fucus and Laminaria. In areas of strong water movement.

ESPICULAS
SPICULES



Megascleras:
1. Estilos ou subtilostilos, pequenos e gordos
2. Subtilostilos finos
Microscleras:
3. Toxas com pontas finas

Megascleres:
1. Styles or subtylostyles, small and fat
2. Subtylostyles thin
Microscleres:
3. Toxa with smooth tips



Tedania pillarriosae

Cristobo,2002

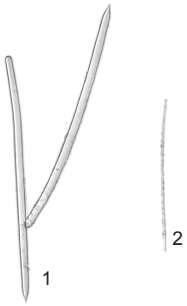
Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Poecilosclerida
Família Family
Tedaniidae

DESCRIÇÃO
DESCRIPTION

Cor: Laranja com tons de castanho na superfície. Interior laranja brilhante. Laranja escuro em álcool.
Forma: Massiva.
Consistência: Firme, pouco compressível, fácil de quebrar.
Superfície: Regular e suave. Pequenas protuberâncias visíveis em alguns locais.
Habitat: Zona intertidal ou sublitoral rasa. Em superfícies rochosas graníticas, em fendas e grutas escuras.

Colour: Orange to orange brown at the surface and bright orange inside. Dark orange in alcohol.
Shape: Massive.
Consistency: Firm, barely compressible, easily torned.
Surface: Even, soft. Small conules visible in some parts.
Habitat: On intertidal or subtidal shallow areas. On rocky granitic surfaces, dark caves, and in crevices.

ESPICULAS
SPICULES



Megascleras:
1. Estilos
(Estronguilos raros ou ausentes)
Microscleras:
2. Oniquetas

Megascleres:
1. Styles
(Strongyles rare or absent)
Microscleres:
2. Onychaetes

Phorbas plumosus
(Montagu, 1818)

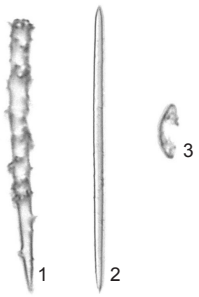
Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Poecilosclerida
Família Family
Hymedesmiidae

DESCRIÇÃO
DESCRIPTION

Cor: Variável de laranja a violeta acastanhado.
Forma: Massiva, mais ou menos espessa, ou em forma de almofada.
Consistência: Compressível, bastante resistente.
Superfície: Mais ou menos macia, ou ligeiramente tuberculada, com numerosos ósculos visíveis, assim como os canais exalantes.
Habitat: Águas rasas, zona de algas.
Outras características: Cheiro intenso.

Colour: Orange to violet-brown.
Shape: Massive, more or less thick, or cushion shape.
Consistency: Compressible, very resistant.
Surface: More or less smooth, or slightly tuberculate, with numerous visible oscules. Excurrent channels also visible.
Habitat: Typically in shallow waters, in the kelp zone.
Other remarks: Strong smell.

ESPICULAS
SPICULES



Megascleras:
1. Acantóstilos em dois tamanhos distintos
2. Tornotes
Microscleras:
3. Isoquelas arqueadas

Megascleres:
1. Acanthostyles in two different sizes
2. Tornotes
Microscleres:
3. Arcuate isochelae

Amphilectus fucorum
(Esper,1794)

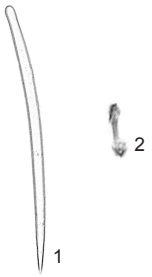
Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Poecilosclerida
Família Family
Esperiopsidae

DESCRIÇÃO
DESCRIPTION

Cor: Vermelho alaranjado (incolor em álcool). Pode ser incolor em águas profundas. Quando espremida liberta pigmento.
Forma: Elevado polimorfismo. Em zonas com pouca corrente pode apresentar longos filamentos.
Consistência: Macia e quebrável, com contração leve.
Superfície: Uniforme. Com pequenos poros exalantes e ósculos dispersos por toda a superfície, podendo emergir ligeiramente, ou situarem-se no topo de projeções em forma de vulcão. Possui uma fina camada transparente e viscosa.
Habitat: Pode ser encontrada em correntes fortes. Sobre rochas. Poderá crescer junto com a alga *Laminaria*, conchas ou ascídios. Típica de águas pouco profundas.
Outras características: Possui cheiro forte e desagradável.

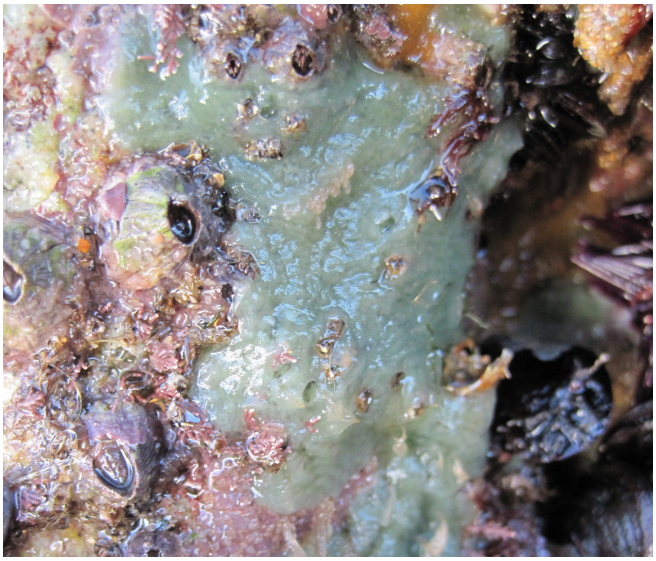
Colour: Orange reddish (colourless in alcohol). Can be colourless in deep waters. When squeezed, a reddish pigment is released.
Shape: Extremely polymorphic. Can present long filaments in areas of low tide.
Consistency: Soft and easily torn. Slight contraction.
Surface: The surface is covered with small exhalant pores and oscules, slightly raised from the surface, or on top of volcano shaped projections. Covered with a thin, transparent and slimy layer.
Habitat: Characteristic from strong tide areas. Appears on rock surfaces. Commonly near the green algae *Laminaria*, shells and ascidians. Occurs normally in shallow waters.
Other remarks: Presence of a strong and unpleasant smell.

ESPICULAS
SPICULES



Megascleras:
1. Estilos lisos e curvados, com tamanho variável
Microscleras:
2. Isoquelas palmadas pequenas (podem ser raras)

Megascleres:
1. Styles smooth and slightly curved
Microscleres:
2. Isochelae palmate small (can be rare)



Halichondria (Halichondria) panicea
(Pallas, 1766)

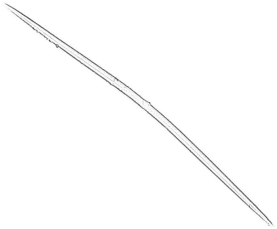
Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Suberitida
Família Family
Halichondriidae

DESCRIÇÃO
DESCRIPTION

Cor: Amarela alaranjada. Esverdeada em locais bem iluminados, possivelvente devido à presença de microssimbiontes.
Forma: Variável; Normalmente incrustante.
Consistência: Compressível e pode facilmente partir-se.
Superfície: Espécimes a crescer em zonas intertidais muito expostas ao mar podem possuir a superfície completamente lisa, quase sem chaminés osculares visíveis. Em zonas mais protegidas, desenvolvem chaminés em forma típica de vulcão, com ósculos relativamente grandes.
Habitat: Trata-se de uma espécie oportunista. Encontra-se nas rochas ou outros substratos duros, como conchas.
Outras características: Forte odor

Colour: Orange-yellow or pale yellowish green. Greener when exposed to sun light, possibly due to the presence of microsymbionts.
Shape: Variable. Normally incrusting.
Consistency: Firm, compressible, easily torn.
Surface: Specimens growing in the intertidal region, exposed to the full oceanic surf may be entirely smooth with barely visible oscular chimneys. More intermediate environments show the typical volcano-shaped chimneys, with oscules relatively large.
Habitat: It is an opportunistic species. Found on rocks and other hard substrates, like shells.
Other remarks: Strong odour.

ESPICULAS
SPICULES



Megascleras:
Oxeas ligeiramente curvadas
Microscleras:
Ausentes

Megascleres:
Oxeas slightly curved
Microscleres:
Absent

Hymeniacidon perlevis
(Montagu, 1814)

Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Suberitida
Família Family
Halichondriidae

DESCRIÇÃO
DESCRIPTION

Cor: Laranja, avermelhada (fica castanho/preta em álcool).
Forma: Tapetes finos, forma de pequenas almofadas ou massivas.
Consistência: Compacta, firme e compressível.
Superfície: Variável, macia, formando tubérculos ou projeções. Ósculos espalhados pela superfície, ao mesmo nível que esta ou no topo dos ramos.
Habitat: Encontra-se em pedras, rochas, conchas. Vários invertebrados encontram-se associados a esta espécie.
Outras características: É a esponja mais comum da costa do Oeste da Europa. Possui um cheiro ligeiramente adocicado.

Colour: Orange, reddish (dark brown.to black in alcohol).
Shape: Thin sheets, cushions, to massive-forms.
Consistency: Firm, compact and compressible.
Surface: Variable, smooth, tuberculate or covered with branching processes. Oscules scattered, at surface level or on top of branching processes.
Habitat: On stones, rocks, shells. Many invertebrates are associated with this species.
Other remarks: The most common species along the coasts of Western Europe. Smell sweetish.

ESPICULAS
SPICULES

Megascleras:
Estilos (podem apresentar duas categorias de tamanho)
Microscleras:
Ausentes

Megascleres:
Styles (can appear in two different sizes)
Microscleres:
Absent

Haliclona sp.

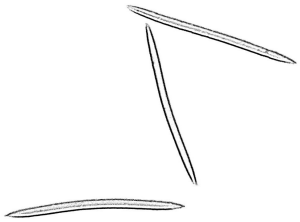
Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Haplosclerida
Família Family
Chalinidae

DESCRIÇÃO
DESCRIPTION

Cor: Entre o branco e o rosa.
Forma: Massiva com fistulas que surgem da zona superior e lateral da esponja. Podem surgir fistulas mais finas e sem ósculos.
Consistência: Firme, ligeiramente frágil.
Superfície: Macia. Nas fistulas surgem ósculos bastante largos.
Habitat: Locais relativamente protegidos mas com movimentação de águas. Mais frequente em substratos verticais que horizontais.

Colour: Whitish to pinkish.
Shape: Massive, commonly with fistules arising from the upper and side parts of the sponge. Thinner fistules, with no oscules may be present.
Consistency: Rather firm, slightly brittle.
Surface: Smooth. Oscules present in the thicker fistules.
Habitat: In fairly sheltered places with moderate water movement. More frequent on vertical than on horizontal substrates.

ESPICULAS
SPICULES



Megascleras:
Oxeas a direito ou ligeiramente curvadas
Microscleras:
Ausentes

Megascleres:
Oxeas, straight or slightly curved
Microscleres:
Absent

Haliclona simulans

(Johnson, 1842)

Classe Class
Demospongiae
Subclasse Subclass
Heteroscleromorpha
Ordem Order
Haplosclerida
Família Family
Chalinidae

DESCRIÇÃO
DESCRIPTION

Cor: Várias escalas de castanho, amarelo, laranja ou cinzento. Áreas em volta dos ósculos são mais esbranquiçadas.
Forma: Extremamente polimórfica. Pode formar tapetes muito finos a grandes massas.
Consistência: Firme, incompressível.
Superfície: Forma extensões com ósculos bem visíveis.
Habitat: Debaixo de rochas e em fendas.

Colour: Various shades of brown, yellow, orange and grey. Areas surrounding the oscula are whitish.
Shape: Extremely polymorphic. From thin sheets to large masses.
Consistency: Hard, uncompressible.
Surface: Form extensions with visible oscules.
Habitat: Under rocks or crevices.

ESPICULAS
SPICULES

Megascleras:
Oxeas
Microscleras:
Ausentes

Megascleres:
Oxeas
Microscleres:
Absent

Dysidea fragilis
(Montagu, 1814)

Classe Class
Demospongiae
Subclasse Subclass
Keratosa
Ordem Order
Dictyoceratida
Família Family
Dysideidae

DESCRIÇÃO
DESCRIPTION

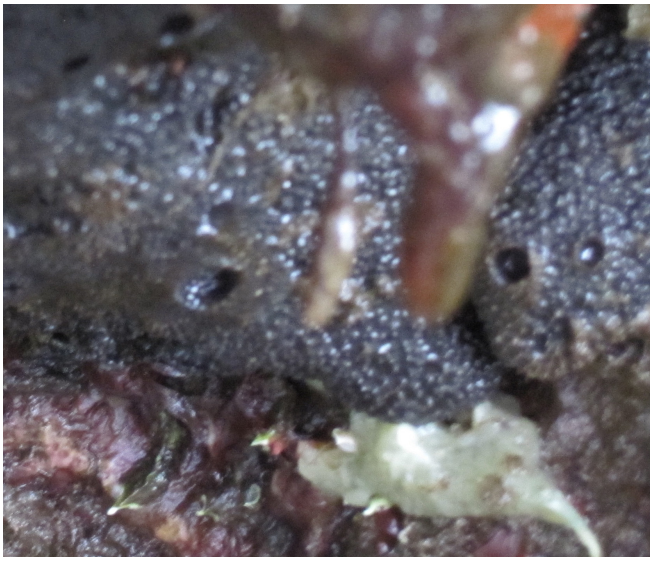
Cor: Esbranquiçada ou cinza. Também pode ser castanha.
Forma: Incrustante ou massiva.
Consistência: Variável. Elástica (dependendo da quantidade de espongina). Normalmente resistente.
Superfície: Macia, formando pequenas estruturas semelhantes a cones. Ósculos dispersos.
Habitat: Rochas, fendas, sedimento, conchas, areia.

Colour: Whitish to grey. Can also be brown.
Shape: Incrusting or lobate.
Consistency: Variable. Elastic (depending on the amount of spongin). Usually tough.
Surface: Smooth and conulose. Oscules scattered.
Habitat: Rocks, crevices, on shells or gravel.

ESPICULAS
SPICULES

Sem espiculas

No spicules



Ircinia variabilis
(Schmidt, 1862)

Classe Class
Demospongiae
Subclasse Subclass
Keratosa
Ordem Order
Dictyoceratida
Família Family
Ircinidae

DESCRIÇÃO
DESCRIPTION

Cor: Variável: cinzento, esverdeado, castanho, esbranquiçado, violeta.
Forma: Variável: incrustante a massiva.
Consistência: Firme. Difícil de partir.
Superfície: Coberta por pequenas estruturas conulosas. Ósculos distribuídos irregularmente e elevados da superfície.
Habitat: Superfícies rochosas, protegidos da luz solar, em cavidades ou grutas.

Colour: Variable: grey, greenish, brown, whitish, violet.
Shape: Incrusting or massive.
Consistency: Firm. Hard to tear or cut.
Surface: Covered with small conules. Oscules scattered throught the surface and slightly elevated.
Habitat: Rocky surfaces, protected from direct sun light, on caves or crevices.

ESPICULAS
SPICULES

Sem espiculas

No spicules

Aplysilla rosea

(Barrois, 1876)

Classe Class
Demospongiae
Subclasse Subclass
Keratosa
Ordem Order
Dendroceratida
Família Family
Darwinellidae

DESCRIÇÃO
DESCRIPTION

Cor: Vermelho rosado.
Forma: Incrustante, formando um tapete muito fino.
Consistência: Suave e compressível.
Superfície: Coberta de pequenas projeções de fibras (efeito "pele de galinha"). Entre as projeções é bastante macia. Com muitos ou apenas 1 ósculo, situado no topo de chaminés oscu-lares.
Habitat: Rochas na zona intertidal. Em zonas de sombra, pro-tegidas.

Colour: Brick or deep red.
Shape: Incrusting and thin.
Consistency: Soft and compressible.
Surface: With projections at the surface forming low conules of protruding single fibbers ("goose flesh" effect). Smooth between conules. With one or more oscules at the top of oscular chim-neys.
Habitat: Common under boulders in the intertidal region. On shaded locations.

ESPICULAS
SPICULES

Sem espiculas

No spicules

Este trabalho foi financeiramente suportado pelo projeto MARBIOTECH - NORTE-07-0124-FEDER-000047 e pelo Governo Português, através da Fundação para a Ciência e Tecnologia (FCT) através dos projetos PesT-C/MAR/LA0015/2011 e PTDC/MAR/099642/2008, a Bolsa de Doutoramento SFRH/BD/73033/2010, e a Bolsa de Investigação BI/PTDC/MAR/099642/2008/2011-030. O trabalho foi desenvolvido no Laboratório Associado, Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), da Universidade do Porto (UP), no Laboratório Blue Biotechnology and Ecotoxicology (BBE).

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NACIONAL
COMPETE
2020

Poster

For scientific divulgation


Esponjas Intertidais do Litoral Norte de Portugal
Intertidal Sponges from the Northern Coast of Portugal
Ana Regueiras, Maria Sofia Costa e Vítor Vasconcelos
C. Calcarea > O. Clathrinida
Clathrina coriacea
C. Demospongiae > O. Suberitida
Aaptos papillata
C. Demospongiae > O. Polymastiida
Polymastia aglutinans
C. Demospongiae > O. Polymastiida
Polymastia penicillus
C. Demospongiae > O. Clionaida
Cliona celata
C. Demospongiae > O. Axinellida
Stelligera rigida
C. Demospongiae > O. Poecilosclerida
Clathria coralloides
C. Demospongiae > O. Poecilosclerida
Ophlitaspongia papilla
C. Demospongiae > O. Poecilosclerida
Tedania pillarriosae
C. Demospongiae > O. Poecilosclerida
Phorbas plumosum
C. Demospongiae > O. Poecilosclerida
Amphilectus fucorum
C. Demospongiae > O. Suberitida
Halichondria panicea
C. Demospongiae > O. Suberitida
Hymeniacidon perlevis
C. Demospongiae > O. Haplosclerida
Haliclona sp.
C. Demospongiae > O. Haplosclerida
Haliclona simulans
C. Demospongiae > O. Dictyoceratida
Dydidea fragilis
C. Demospongiae > O. Dictyoceratida
Ircinia variabilis
C. Demospongiae > O. Dendroceratida
Aplysilla rosea
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COMPETE

Brochure

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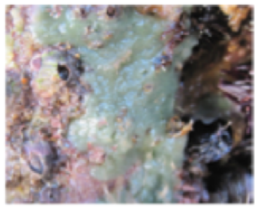
Ordem Order — **SUBERITIDA**

Halichondria panicea



Amarela alaranjada. Esverdoada em locais bem iluminados. Compressível. Superfície lisa quando exposta ao mar, ou com chaminés em forma de vulcão em zonas protegidas. Ósculos grandes. Surge em rochas. Com odor forte.

Orange-yellow. Greener when exposed to sunlight. Compressible. Surface smooth when exposed to full oceanic surf or with volcano-shaped chimneys in more intermediate environments. Large oscules. Found on rocks. Strong odour.

Hymeniacidon perlevis

Laranja, avermelhada. Compacta, firme e compressível. Superfície variável, macia, com projeções. Ósculos espalhados pela superfície. Encontra-se em pedras, rochas, conchas. Possui um cheiro ligeiramente adocicado.

Orange, reddish. Firm, compact and compressible. Surface variable, smooth, covered with branching processes. Oscules scattered. On stones, rocks, shells. Smell sweetish.

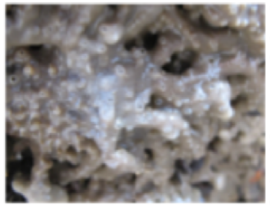
Ordem Order — **HAPLOSCLERIDA**

Haliclona sp.

Branca a rosa. Forma massiva com projeções (fístulas) onde se encontram os ósculos. Firme, ligeiramente frágil e macia. Surge em locais protegidos mas com movimentação de águas.

From whitish to pinkish. Shape is massive, commonly with fistules, where oscules are present. Rather firm, slightly brittle and smooth. Common in sheltered places with moderate water movement.

Haliclona simulans

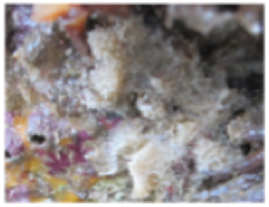


Castanho, amarelo, laranja ou cinzento, mais claro em volta dos ósculos. Forma tapetes finos ou grandes massas, com extensões com ósculos visíveis. Firme, incompressível. Comum debaixo de rochas e em fendas.

Brown, yellow, orange or grey, whitish on areas surrounding the oscula. From thin sheets to large masses. Hard, uncompressible. Form extensions with visible oscules. Common under rocks or crevices.

Ordem Order — **DICTYOCERATIDA**

Dysidea fragilis



Esbranquiçada, cinza ou castanha. Forma incrustante ou massiva. Elástica, normalmente resistente. Macia, formando pequenas estruturas semelhantes a cones. Ósculos dispersos. Surge em rochas, fendas, sedimento, conchas ou areia.

Whitish to grey, or brown. Incrusting or massive. Elastic, usually tough. Smooth and conulose. Oscules scattered. Appears on rocks, crevices, on shells or gravel.

Ircinia variabilis

Cinzento, esverdoado, castanho, esbranquiçado, violeta. Firme, difícil de partir. Coberta por pequenas estruturas cónulosas. Ósculos distribuídos irregularmente e elevados da superfície. Em superfícies rochosas, protegidos da luz solar.

Grey, greenish, brown, whitish, violet. Firm, hard to tear or cut. Covered with small conules. Oscules scattered through the surface and slightly elevated. On rocky surfaces, protected from direct sunlight.

ESPONJAS (PORIFERA) DO INTERTIDAL DA COSTA PORTUGUESA

INTERTIDAL MARINE SPONGES (PORIFERA) FROM THE PORTUGUESE COAST

ciimar

CRÉDITOS
CREDITS

ANA REGUEIRAS
VÍTOR VASCONCELOS

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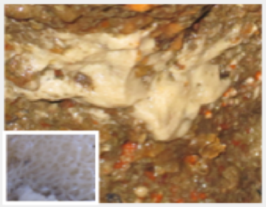
Divulgação no âmbito do projeto MARBIOTECH e NOVOMAR
Outreach activity from project MARBIOTECH and NOVOMAR

Classe Class — CALCAREA

Ordem Order — CLATHRINIDA

Clathrina coriacea

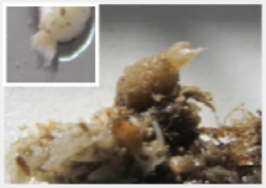


Branca a amarelo pálido. Forma de "almofadas", Com estrutura em forma de treliça, compressível e suave. Ósculos ligeiramente elevados da superfície. Surge em rochas ou fendas.

White to pale yellow. Shape of small cushions, formed by a tightly knit trelliswork of tubes. Smooth and compressible. Oscules slightly elevated from the surface. Appears under boulders or crevices.

Ordem Order — LEUCOSOLENIDA

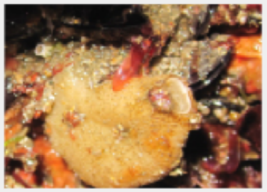
Sycon ciliatum



Esbranquiçada. Com forma tubular. Surge individualmente ou em pequenos grupos. Superfície pilosa. Com ósculo terminal, rodeado por franja de espículas. Suave e firme. Surge em rochas.

Whitish. With a tubular shape. Appears normaly alone, or in small groups. Hairy surface. With a terminal oscule, surrounded by a fringe of spicules. Soft but firm. Appears on rocks.

Grantia compressa



Branca a beje. Forma massiva, lobada, ou incrustante. Superfície ligeiramente rugosa, com ósculos no topo dos lóbulos. Firme, frágil. Surge em rochas.

White to beije. Can be massive, lobed or incrusting. Surface slightly rough, with oscules on top of the lobes. Firm, bristle. Appears on rocks.

Classe Class — DEMOSPONGEAE

Ordem Order — SUBERITIDA

Aaptos papillata

Violeta com extremidade das pápilas claras. Interior alaranjado. Forma hemisférica ou de almofada. Superfície hispida com numerosas pápilas. Firme e difícil de retirar do substrato. Enterrada na areia, apenas pápilas visíveis à superfície.

Shades of violet, lighter at papillae tips. Orange internally. Hemispheric or pillow shape, slightly hispid with numerous papillae. Firm, hard to remove from the substrate. Buried on the sand, detectable only by the papillae sticking out.

Ordem Order — POLYMASTIIDA

Polymastia agglutinans

Amarelo a laranja. Forma de almofada, com papilas que se projetam do corpo, enterrado no substrato. O corpo e as papilas são duros. Papilas longas e finas. Ósculos encontram-se nas extremidades das papilas. Particulas incrustadas à superfície. Sob camada de sedimentos

Yellow to orange. Shape of cushion with papillae projecting from the sediment covered body. Body and papillae hard. Thin and long papillae. Oscules on the papillae tips. Foreign material incrusted to the surface On sandy bottoms.

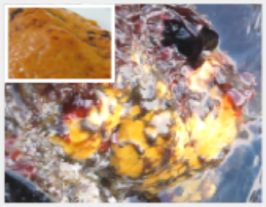
Polymastia penicillus

Amarelo. Forma de almofada, com papilas que se projetam do corpo, enterrado no substrato. O corpo e as papilas são duros. Ósculos encontram-se nas extremidades das papilas. Surge sob camadas de sedimentos.

Yellow. Shape of cushion with papillae projecting from the sediment covered body. Body and papillae hard. Oscules on the papillae tips. On sandy bottoms.

Ordem Order — CLIONAIDA

Cliona celata

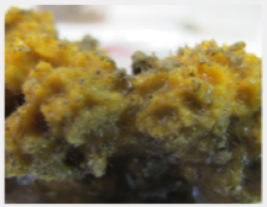


Amarela. Escurece fora de água. Forma massiva com papilas caracteristicamente achatadas. Compacta e firme, com superfície macia e coberta por poros inalantes retrácteis. Com ósculos grandes. Surge em rochas.

Yellow, becoming darker outside water. Sponge large massive, with characteristic flattened papillae. Firm, compact and smooth surface. Covered with inhalant retractable papillae. With big oscules. Occurs on rocks.

Ordem Order — AXINELLIDA

Stelligera rigida



Amarelo pálido a laranja. Firme, corpo ramificado com extremidades em forma de bolbos. Superfície hispida, ouriçada. Ósculos pequenos. Surge em locais abrigados mas com alguma corrente.

Pale yellow to orange. Firm, branching-erect, with bulbous-like extremities. Surface strongly hispid, bristly. With small oscules. Appears in sheltered locations with some current.

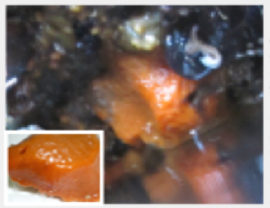
Ordem Order — POECILOSCLERIDA

Ophlitaspongia papilla

Laranja a vermelho forte. Quando espremida liberta pigmento. Forma finos tapetes ou almofada. Forte e compressível. Parte-se com uma bolacha pouco dura. Superficie hispida, com numerosos ósculos. Surge em rochas.

Bright orange-red. Pigment is released when squeezed. Forms thin sheets or cushions. Firm and compressible. Brakes like a soft cookie. Surface hispid, with numerous oscules. Appears on rocks.

Tedania pillarriosae



Laranja a castanho. Interior laranja brilhante. Forma massiva. Firme, fácil de quebrar. Superficie regular e suave, com pequenas protuberâncias visíveis. Surge em superficies rochosas, protegida da luz.

Orange to orange brown at the surface and bright orange inside. Firm, and easily torned. Surface even and soft. Small conules visible in some parts. On rocky surfaces, dark caves, and in crevices.

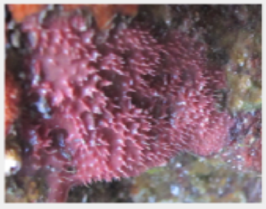
Phorbas plumosus

Laranja a violeta acastanhado. Forma massiva. Compressível, bastante resistente. Superficie ligeiramente tuberculada, com numerosos ósculos visíveis. Surge em águas rasas, zona de algas. Possui cheiro muito intenso.

Orange to violet-brown. Massive. Compressible, very resistant. Surface slightly tuberculate, with numerous visible oscules. Typically in shallow waters, in the kelp zone. With a strong odour.

Ordem Order — DENDROCERATIDA

Aplysilla rosea



Vermelho rosado. Forma tapete muito fino. Suave e compressível. Coberta de pequenas projeções de fibras (efeito "pele de galinha"). Com muitos ou apenas um ósculo. Presente em rochas, em zonas protegidas.

Brick or deep red. Extremely thin. Soft and compressible. With projections forming low conules of protruding single fibbers ("goose flesh" effect). With one or more oscules. Common under boulders.